

**Methods Development for Isolation of Carbon Nanomaterial Degradation Products Under
Simulated Conditions Relevant for their Proposed Use in Desalination Membranes**

by

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University of Pittsburgh, 2019

Membrane desalination is a crucial process that enables access to unconventional drinking water sources, such as brackish water and seawater. Membranes are used in the desalination process and critical challenges remain for optimizing performance, most notably biofouling. New innovations in desalination membranes that involve the incorporation of emerging nanomaterials, including carbon nanotubes (CNTs) and graphene, propose a potential solution to alleviate biofouling as well as enhance salt and contaminant rejection. However, carbon nanomaterials (CNMs) have been shown to degrade through chemical and biochemical pathways. While all degradation products have not been identified under all studied conditions, existing literature suggests the possible formation of polycyclic aromatic hydrocarbons (PAHs) and their derivatives. The complete degradation pathways of forming these or similar degradation products (DPs) are not resolved under relevant desalination conditions, including the presence of key components needed to catalyze CNM degradation in these processes, namely relevant enzymes (e.g., from biofilm) and oxidants (e.g., from membrane cleaning). The potential to produce harmful DPs is of concern due to the possible subsequent release to drinking and irrigation waters. This research develops an experimental approach to identify the formation of DPs under simulated desalination conditions as an initial step towards determining their potential formation in desalination processes that use CNM-enabled membranes. Single wall carbon nanotubes (SWCNTs) with characteristics matching those used most in the membrane literature were exposed to model conditions containing

various representative chemicals used as membrane cleaning agents (sodium hydroxide, ethylene diamine tetraacetic acid, and sodium dodecyl sulfonate) and *Sphingomonas* biofilm. *Sphingomonas aromaticivorans* was chosen because it has been identified as the dominant species that also initiates biofilm formation on RO membranes and thus, would be in direct contact with the CNT-amended membrane. This study investigated and simulated membrane desalination conditions and developed methods for CNM DP isolation. High performance liquid chromatography (HPLC) was used to characterize degradation of parent PAH molecules and SWCNTs under different conditions. The results herein serve as the foundation for future research to further resolve and identify the chemical structure of DPs with the ultimate goal of safely realizing the potential of the novel CNM-enabled membranes.

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Preface

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Abbreviations

The nomenclature used in this document are listed as follow.

ATCC American Type Culture Collection	LC-MS/MS Liquid Chromatography Tandem Mass Spectrometry
CNM Carbon Nanomaterial	MSM Mineral Salt Medium
CNT Carbon Nanotube	MWCNT Multi Wall Nanomaterial
CVD Chemical Vapor Deposition	PAH Polycyclic Aromatic Hydrocarbon
DP Degradation Product	rGO Reduced Graphene Oxide
EDTA Ethylene Diamine Tetra-Acetic Acid	RO Reverse Osmosis
EPA Environmental Protection Agency	RT Retention Time
EPS Extracellular Polymeric Substances	SDS Sodium Dodecyl Sulfonate
FT-IR Fourier Transform Infrared Spectroscopy	SWCNT Single Wall Nanomaterial
GC-MS Gas Chromatography-Mass Spectrometry	TEM Transmission Electron Microscopy
GO Graphene Oxide	TGA Thermal Gravity Analysis
HPLC High Performance Liquid Chromatography	TPH Total Petroleum Hydrocarbons
LC-MS Liquid Chromatography-Mass Spectrometry	TS Tryptic Soy
	UV-Vis Ultraviolet Visible Spectroscopy
	XPS X-Ray Photoelectron Spectroscopy

1.0 Introduction

Two thirds of the global population is facing severe drought at least one month of the year due to the complex and rapidly changing geography of the water supply caused by climate change and population development^{1, 2}. Although seawater covers 71% of the Earth's surface and represents more than 97% of the world's water, most of it is unusable for industry, agriculture, and drinking purposes. Together with the steadily increasing consumption of freshwater², anthropogenic activities and pollutants have exacerbated disruptions of the water cycle^{1, 3}, thus motivating communities across the world to seek solutions and alternative water resources for drinking and irrigation usage. As one feasible solution, desalination is a crucial technology that enables access to unconventional drinking water sources by removing salts and minerals from high-salinity brackish water and seawater⁴⁻⁶. At the same time, membrane processes, such as reverse osmosis, are the desalination technology most widely used in recovery of high-quality fresh water^{7, 8}. Despite the wide application of these membrane-based water treatment technologies, critical challenges remain for optimizing performance. Biofouling is a primary challenge and can lead to a higher operating pressure, flux decline, frequent chemical cleaning, and shorter membrane life⁸⁻¹⁰. As a result, biofouling necessitates pretreatment and frequent replacement of the membrane unit.

Recently, the incorporation of carbon nanomaterials (e.g., carbon nanotubes¹¹⁻¹⁶ and graphene¹⁷⁻²⁰) into desalination membranes has been proposed as a potential solution to biofouling²¹ and to enhance salt and contaminant rejection²². However, potential adverse consequences associated with the use of carbon nanomaterials (CNMs) in desalination membranes have received much less attention. Numerous studies indicate that CNMs degrade via chemical

and biochemical pathways, forming degradation products (DPs) such as polycyclic aromatic hydrocarbons (PAHs)²³⁻³⁰. Although the potential formation of these DPs or their derivatives has not been studied under relevant desalination conditions, the existence of similar chemical and biological constituents (e.g., bacteria, oxidants, and hydrocarbons) in these processes suggests that their formation is possible. PAHs are a class of widely distributed chemicals that are composed of multiple aromatic rings. Certain PAHs, such as benzo[a]pyrene, are highly hazardous to human health due to a number of carcinogenic and immunosuppressant substances generated within their degradation process³¹⁻³³. The DPs of these harmful PAHs have structures similar to those empirically observed from degradation of CNMs under relevant conditions^{23, 34-36}. Given that the toxicity of PAH transformation products depends strongly on structure, a comprehensive investigation of the potential for generation of hazardous DPs from CNM-amended membranes and of their subsequent release in drinking and irrigation waters is necessary^{24, 37-42}. To assess the potential risk of CNM-DPs and elucidate its degradation pathway, fundamental research on simulating water chemistry presented in membrane desalination conditions and developing isolation methods of CNM DPs was conducted.

To simulate the relevant membrane desalination conditions, model biological and chemical constituents were selected based on previous studies. *Sphingomonas spp.* is considered as a major microorganism that leads to membrane fouling via its ability to initiate biofilm formation and dominate the microorganism community⁹. The genus *Sphingomonas* is capable of surviving under oligotrophic conditions and can metabolize a great variety of organic sources^{26, 43-45}. Combined, these properties of *Sphingomonas* allow them to adapt to environments with minimal nutrients and complex ingredients needed for growth, similar to desalination conditions. *Sphingomonas* is widespread in the environment and has been reported to exist in diverse RO desalination

facilities^{46, 47}. Thus, it is used in this study as the model biofilm. Moreover, a variety of chemical agents (e.g., H₂O₂, NaOH, EDTA, and SDS)^{48, 49} are involved in the membrane cleaning process, and have chemical properties are similar to those applied in CNM-degradation studies (e.g., oxidants, bases, and surfactants)^{25, 29, 35, 36, 50}. This suggests their potential in promoting CNM degradation and thus, their effect on the water chemistry and interaction with microorganism should be considered.

To assess the potential risk of CNM-DPs, an experimental approach is used to isolate and detect the DPs from SWCNTs, chosen because they are a predominant CNM structure found in the membrane literature. SWCNTs are exposed to simulated conditions containing chemical and biological components representative of RO membrane processes. The potential formation of DPs under these conditions are analyzed using high performance liquid chromatography (HPLC).

1.1 Objectives

The current research focuses on establishing experimental approaches to evaluate the potential for CNMs to form DPs in environments commensurate with membrane desalination, specifically RO processes. The goal of this research is to identify the conditions under which CNMs degrade and determine whether DPs are generated, thus serving as a foundation for future research on understanding the potential risks of using CNMs in desalination membrane processes. The long-term goal of this research is to inform the design of next-generation membranes and to guide the safe use of promising CNM-enabled technologies.

The specific objectives for this work include the following:

1. Identify the primary conditions (biofoulants, chemical foulants, and cleaning agents) of the RO membrane desalination, defining the parameters for our study to determine if DPs form.
2. Develop a representative biofilm system, including the conditions that CNM-enabled membranes encounter during their lifetime, and investigate the effect of the biofilm on CNM DP under different nutrient conditions.
3. Develop DP isolation and HPLC detection methods..
4. Determine CNM DP formation in representative RO treatment conditions, including the presence of microorganisms or membrane cleaning reagents.

1.2 Approach

In this study, DP formation from SWCNT was studied under simulated conditions relevant to RO desalination. To represent the desalination process conditions, biological foulants were first investigated to identify the biofilm composition, particularly those at the base, which will be in contact with the CNT-modified membrane. The identified microorganism was used to develop an artificial biofilm system to study the interaction with SWCNTs. In addition to pristine SWCNTs, pre-treated samples were prepared through exposure to representative membrane cleaning agents, prior to exposure to the biofilm. At determined time points, CNM DPs were isolated from the system and were characterized using HPLC. Potential DPs were isolated by two distinct methods: i) extracted by organic solvent and concentrated with rotary evaporator; ii) directly injected into analytical instrument after centrifugation to remove any insoluble particles. The isolation methods were further verified and compared with representative PAHs. For future study, the qualitative

results from HPLC could be used to indicate whether further resolution is required using liquid chromatography tandem mass spectrometry (LC-MS/MS) due to its advantages of increased sensitivity and capacity to gain more detailed structural information^{29, 37}.

2.0 Literature Review

2.1 Desalination

Desalination is a technology used to remove soluble particles from brackish water or seawater to secure greater supplies of water for human consumption or agriculture uses. Application of desalination is growing rapidly due to an increasing scarcity of freshwater, driven by climate change, projected population growth, and water pollution^{1, 2, 51}. Since unconventional water sources account for approximately 97.5% of the entire hydrosphere, capture of a small fraction could have a great improvement on water scarcity⁵². Thus, the challenges and opportunity for addressing such problem are of great interest to scientists for research on this field.

2.2 Reverse Osmosis

Thermal and membrane processes are the major practices of current desalination technology^{5, 6, 8, 22}. Due to the high energy requirement, large footprint requirement, rapid scale formation, and corrosion, thermal desalination is limited to regions that have access to notably large amounts of heat. However, membrane processes such as reverse osmosis (RO) are widely used because they are more compact and less energy-intensive than the thermal process^{7, 22}. With the advantage of a notably high salt rejection rate (greater than 99%), the installation of RO desalination is steadily increasing. In 2019, the global projected amount of desalinated water is

approximately 34.8 billion m³ per year, double the record from decade years ago^{53, 54}. In the RO desalination process, saline water is withdrawn, pretreated to remove any suspended solids, and pumped to a device that consists primarily of a selective semipermeable membrane. The fine pore size on the membrane rejects ions and other organic compounds, while allowing fresh water to pass through. As a critical underpinning of desalination, membrane technologies face significant challenges such as energy use, operational cost and environmental impacts^{8, 55}. Biofouling and maintenance deteriorate the membrane performance over time, resulting in membrane damage and frequent replacement of membrane units. Since the membrane is the most crucial component that determines the efficiency for desalination, improvements delivered by CNMs are expected to significantly benefit the entire process.

2.3 Biofouling

Biofouling refers to the process of microorganism growth and adhesion on the membrane surface and is considered a major obstacle for membrane performance due to the irreversible consequences of increased transmembrane pressure and decreased water and salt rejection⁵⁶. In addition, the formation of biofilm is essential in the biofouling process. Although the bacterial community could differ depending on the environment, biological deposition and extracellular polymeric substances (EPS) excreted by the bacteria are the main components of biofilms. The development of biofilm on the membrane involves three stages: bacteria attachment, reproduction, and detachment¹⁰. The first stage is considered the most important because the bacteria that are first attached to the membrane can excrete EPS during proliferation, which supplies a favorable environment for the following microorganisms to attach and functions as a barrier to protect the

microorganisms^{10, 56}. Although many bacteria participate in biofilm formation on the RO membrane, *Sphingomonas* species was found to be the primary organism in biofouling⁹. The genus *Sphingomonas* is capable of surviving under oligotrophic desalination conditions, can metabolize a great variety of organic sources, is widely found in the environment, and has been identified in diverse RO desalination facilities^{46, 47}. In the early stage of biofouling, *Sphingomonas spp.* colonizes and rapidly disperses across the RO membrane, modifying the surface with extensive EPS. Therefore, this microorganism is responsible for biofouling initiation. Additionally, the genus *Sphingomonas* is typically located at the bottom layer of the biofilm, which ensures that it survives the cleaning process⁵⁶. In this study, *Sphingomonas spp.* is chosen as the model organism due to its important role in initiating and dominating the biofilm in RO desalination conditions.

2.4 Membrane Cleaning Agents

Over time, the process efficiency of desalination membranes is adversely affected by limescales and biofilm built up on water treatment membranes and eventually require aggressive cleaning to reestablish performance. A variety of chemical agents (as mentioned, H₂O₂, NaOH, EDTA, and SDS, but not limit to these) are involved in this cleaning process, which might promote CNM degradation individually or when combined with the established biofilm. Certain chemicals might serve as oxidants and attack the CNM, resulting in defect sites. For instance, horseradish peroxidase³⁴, eosinophil peroxidase²⁴, myeloperoxidase³⁸ and many other enzymes are able to degrade CNM with the presence of an oxidant. In addition, studies of membrane cleaning indicate a higher permeability recovery rate for in situ chemical cleaning²¹, which increases the potential for CNM degradation due to the existence of residual oxidants. In addition, surfactants such as

sodium dodecyl sulfonate (SDS) are also applied for membrane cleaning. However, surfactants are proven to accelerate the degradation process of PAHs by helping bacteria to contact insoluble aromatic compounds^{26, 33}, and thus membrane cleaning reagents should be considered one of the potential factors that drive the CNM degradation process.

Table 1 lists the most prevalent and emerging chemical cleaning reagents together with their cleaning mechanisms and characteristics²¹.

Table 1 Mechanisms and characteristics of the most prevalent and emerging chemical cleaning agents for membranes. Information is compiled from Ref 21.

Class	Agent	Target Foulant	Advantages	Limitations
Acid	HCl	Inorganic foulants	Effective, low cost	pH shock, membrane damage
	HNO ₃			
	H ₃ PO ₄		Maintain pH	Contribute to TP at effluent
	Citric		Maintain pH, more effective at removing metal cations compared with mineral acids	Higher cost compared with mineral acids
	Oxalic			
Base	NaOH	Organic foulants	pH regulation	Extreme alkaline
Oxidant	NaOCl	Organic and biological foulants	Effective	Membrane damage, produce toxic by-products
	H ₂ O ₂		Do not produce toxic by-products	Membrane damage
Other Chemicals	EDTA	Organic foulants associated with metal ions	Effective	Non-biodegradable
	SDS			Foam formation

As a strategy for RO membrane fouling control, chemical cleaning reagents are commonly used to remove organic foulants and limescales. However, introduction of these compounds could cause material defects and promote CNM degradation, consequently damaging the membrane⁴⁸. The cleaning reagents include oxides, organics acids, surfactants and many other harsh chemicals¹⁰. Pre-treatments of SWCNTs was performed to mimic the chemical cleaning process for RO membranes with commonly used agents, their concentrations, and operational conditions.

Hydrogen peroxide is a conventional disinfectant, which is dosed according to the amount required for cleaning procedures⁵⁷. In Mohammadi's study of membrane fouling, sodium hydroxide, ethylene diamine tetra-acetic acid (EDTA), and sodium dodecyl sulfonate (SDS) were found effective for removal of membrane foulants, especially when combined with EDTA or SDS with alkaline solutions⁴⁸. Another study conducted by Ang et al. compared commercial membrane cleaning agents under different temperature, flux rate, and concentration values⁴⁹.

2.5 Carbon Nanomaterials (CNMs)

Recent advances in material science have highlighted CNMs as a potential game-changer for membrane water treatment applications. Pioneering studies of CNM-enabled membranes, including CNT and graphene, demonstrate high rejection of salts and contaminants and antimicrobial properties, which enable enhanced performance and extended membrane lifetime¹⁰.

22, 58-60.

2.5.1 Application of CNMs in RO Membranes

Many studies focus on applying CNMs on the RO membrane for better performance. Bhadra et al. demonstrated a 72% increase in the permeate flux of a carbon nanotube-immobilized membrane compared with the traditional polypropylene hollow fiber membrane. Their research further indicated an even higher permeate flux increase of 114% in carboxylated (means adding carboxyl groups) CNT-immobilized membranes while maintaining a salt reduction rate of 99%⁶¹. These results are matched with a similar study, which indicates a 1.85x and 15x increase in flux and salt reduction for the CNT enhanced membrane⁶². The approach of those researchers is to alter the method of water and membrane interaction by changing the polarity of the surface. Several studies indicate that CNM-enabled membranes have a higher fouling resistance. Vatanpour and Zoqi applied carboxylated CNT to commercial polyamide RO membranes and found less fouling in the modified membrane⁵⁸. Similarly, Tiraferri et al. noted that the onset of biofouling was delayed on a single-walled carbon nanotube (SWCNT)-bonded membrane⁶³.

In addition, distinguishing characteristics such as antimicrobial ability, hydrophilicity, and chemical resistant properties of graphene, graphene oxide (GO), and reduced graphene oxide (rGO) have led to development of next-generation desalination membranes designed to alleviate membrane biofouling²¹. For instance, Choi et al. coated polyamide thin-film composite membranes with a GO nanosheet⁶⁰ and found that the nanosheet serves as a dual-functional protective layer that improves chlorine resistance and the antibiofouling property. The same results were obtained by Kim's group¹⁷, who noted that the polyamide RO membrane containing a mixture of CNT and graphene had better performance on water treatment in terms of water flux, chlorine resistance, long-term durability and mechanical properties due to the unique properties of the CNMs. Moreover, Liang et al. synthesized a GO-polyacrylonitrile membrane and indicated its

outstanding performance on high-salinity water (salt concentration > 100,000 ppm)⁶⁴. The GO-polyacrylonitrile membrane can achieve a high water flux of up to 65.1 L m⁻² h⁻¹ with a rejection rate of 99.8% for desalination purposes.

Therefore, based on the above studies, the development of CNM-enabled membranes delivered significant opportunities to address the environmental impacts posed by conventional desalination technology. However, the extended CNM-enabled membranes' life span implicates they have more opportunities to encounter with environmental foulants and a higher risk to be affected.

In this study, SWCNT is chosen as the target CNM for study due to its prevalence in the CNM-modified membrane literature. SWCNTs share the same single-layer structure with graphene and the same 1-dimensional tube shape with other types of carbon nanotubes, such as multiwalled CNTs (MWCNTs). The dimension and concentration of SWCNTs used in membrane modification studies are summarized in Table 2. Based on this information, a short length (1-5 μm) SWCNT with a diameter of 1-2 nm is most commonly used in the relevant studies. However, the dosage varies from 2 mg/L to 1000 mg/L. It should be noted that the concentration of SWCNT are stock solutions found in the literature, not the final concentration per membrane area. Thus, the concentration that bacteria will interact with will be lower.

Table 2 Dimension, concentration and purity of SWCNTs stock solutions used in the membrane modification literature.

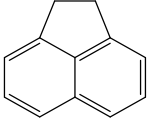
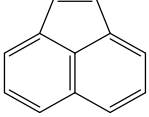
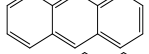
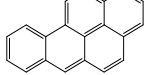
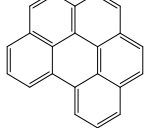
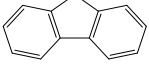
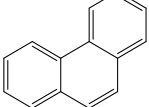
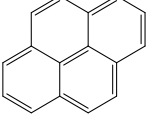
Concentration	Diameter	Length	Purity (%)	Note	Ref
1 g/l	<2 nm	1-5 μ m	>95		65
N/A	0.81 nm	1.34 / 2.7 nm	N/A	Theoretical study	66
N/A	0.81 nm	1.34 nm	N/A	Theoretical study	16
0.04 g/L	0.8 +- 0.1 nm	N/A	>90		63
0.125 g/L	N/A	N/A	N/A	Raw Grade SWCNT	67
0.125 g/L	4-5 nm	~1.0 μ m	94.3		68
2mg/L	N/A	N/A	N/A	¹⁴ C SWCNT	69
0.15 g/L	N/A	0.5-2.25 μ m (near 50% at 1.5 nm)	N/A	SWCNT was oxidized by acid	24
0.03 g/L	90.3+-41.9 nm	1254+-479 nm	N/A	40 min-oxidized SWCNT	70
1 g/l	N/A	N/A	N/A		71
0.1 g/L	1 - 2 nm	0.5 - 2 μ m	90		72
10 mg/L	N/A	129-145 nm	N/A	SWCNTs are modified	73
0.1 g/L	1.42 / 1.52 nm	1 μ m	N/A	o-SWCNT	74
<0.125 g/L	1.3 nm	1 - 2 μ m	N/A		34

2.5.2 Possible Adverse Consequences for CNM Degradation

PAHs and their derivatives are observed in both CNT and graphene degradation studies^{23, 34, 36}. PAHs are a class of chemicals that are composed of multiple aromatic rings. These compounds are widely distributed in the environment and are considered environmental pollutants. Although anthropogenic activities (e.g., combustion of coal, oil, gas, wood, garbage, and tobacco) are the main source of PAHs and their derivatives, these compounds can be prevalently generated by incomplete combustion of organic matter⁴⁴. Certain PAHs pose hazards to human health due to a number of carcinogenic and immunosuppressant substances generated within their degradation

process³¹⁻³³. For example, the five-ring PAH benzo[a]pyrene is a carcinogen⁷⁵. PAHs are readily absorbable from the gastrointestinal tract of mammals and can be rapidly distributed in a wide variety of tissues, especially those rich of body fat. Relevant studies confirmed that exposure to PAH not only increases the probability of various organ cancers but also affects the hematopoietic, immune, reproductive, and neurologic systems and causes developmental defects³¹. In fact, PAHs are classified as priority pollutants and are strictly restricted by the US Environmental Protection Agency (USEPA). Table 3 shows the eight PAHs listed on the EPA Priority Chemical list.

Table 3 Physical and chemical properties of PAHs listed as USEPA priority chemicals for a subset selected from total 21 PAHs from EPA's list.

PAHs	CAS No.	Chemical formula	Chemical structure	Molecular weight (g/mol)	Melting point (°C)	Boiling point (°C)	Density (g/cm ³)	Solubility in water (20°C)
Acenaphthene	83-32-9	C ₁₂ H ₁₀		154.21	95	96.2	1.222	40 mg/100 ml
Acenaphthylene	208-96-8	C ₁₂ H ₈		152.2	92-93	265-275	0.8987	Insoluble
Anthracene	120-12-7	C ₁₄ H ₁₀		178.23	218	340	1.25	Insoluble
Benzo(a)pyrene	50-32-8	C ₂₀ H ₁₂		252.09	176.5	495	1.4	Insoluble
Benzo(g,h,i)perylene	191-24-2	C ₂₂ H ₁₂		276.3307	278	500	1.378	2.6 x 10 ⁻⁴ mg/L
Fluorene	86-73-7	C ₁₃ H ₁₀		166.223	116-117	295	1.202	1.992 mg/L
Phenanthrene	85-01-8	C ₁₄ H ₁₀		178.23	101	332	1.18	1.6 mg/L
Pyrene	129-00-0	C ₁₆ H ₁₀		202.25	145-148	404	1.271	0.135 mg/L

Although the generation of PAHs and their derivatives are confirmed in many studies, the potential formation of these degradation products has not been studied under the relevant water treatment and desalination conditions. The similar chemical and biological constituents present in water treatment processes suggest the potential formation of PAHs and their derivatives.

2.5.3 CNM Degradation

The improved performances shown by CNM-enabled membranes are found in numerous research studies. Nevertheless, potential adverse consequences associated with CNM application in membranes have received much less attention. Both CNTs and graphene can degrade through chemical and biochemical pathways, forming PAH derivatives and therefore, potentially harmful DPs^{23, 26, 34, 35, 37, 76}. In the past decades, numerous studies have explored the biological degradation of CNTs, graphene, and their derivatives. Generally, these materials can be degraded via biological metabolism or enzyme catalysis. Table 4 offers a summary of microorganisms that have been found to degrade these CNMs³⁷.

Table 4 Microorganisms capable of degrading CNTs, graphene, and their derivatives ³⁷. Information is compiled from Ref 37.

Microorganism	Taxonomy	Material
Naphthalene-degrading bacteria	Bacteria	GO, graphite, and RGO
A bacterial community comprising <i>Burkholderia kururiensis</i> , <i>Delftia acidovorans</i> , and <i>Stenotrophomonas maltophilia</i>	Bacteria	MWCNTs
<i>Trabusiella guamensis</i>	Bacteria	MWCNTs
<i>Sparassis latifolia</i>	Fungi	SWCNTs
White-rot fungi (<i>Phanerochaete chrysosporium</i>)	Fungi	SWCNTs, oxidized and reduced GRA nanoribbons
<i>Trametes versicolor</i> and natural microbial cultures	Fungi	SWCNTs

Additionally, membrane cleaning reagents such as oxidants and surfactants can interact with CNMs and exacerbate the degradation process^{24, 39, 49}. As a strategy for RO membrane fouling control, chemical cleaning reagents are widely used to remove organic foulants and scales. However, it is possible for those chemicals to react with CNMs on the membranes. For example, Feng et al. studied few-layer graphene degradation under the coexistence of Fe⁺ and hydrogen peroxide³⁵. The few-layer graphene is oxidized by hydrogen peroxide and ultimately transformed into carbon dioxide.

The key components needed to catalyze this degradation (e.g., bacteria, oxidants and hydrocarbons), are present in these emerging next-generation membrane processes, suggesting their potential to produce hazardous DPs and subsequently release them into drinking and irrigation water.

2.5.3.1 Degradation Time

Empirically, a relatively long time is required for CNM degradation than PAHs, from a couple of days to months. For example, Zhang et al. observed 70% degradation of MWCNT using a bacterial community (selected and purified from soil extraction, containing *Phanerochaete chrysosporium*, *Trametes versicolor* or *Pycnoporus* sp.) with a period of 10 days²³. Bai et al. achieved complete degradation of GO in 3 days via the photo-Fenton reaction³⁶. Their results confirmed that the formation of oxidized products during GO degradation may demonstrate toxicity. Over a longer period, Russier et al. reported that 30 days are required for SWCNT degradation, and 60 days are needed for MWCNT degradation with horseradish peroxidase in the presence of hydrogen peroxide²⁵. The degradation of CNM is confirmed by those studies by identifying significant changes on the starting materials (or completely undetectable) and production of DPs. While the previous studies suggest degradation of CNMs within days to weeks, it is dependent on the specific system conditions and relevant to this research, the incorporation into the membrane matrix. Still, the lifetime of commercial membrane is usually around 3 years⁷⁷ such that at least partial degradation is likely.

2.5.3.2 Degradation Agent

Although enzyme-catalyzed CNM degradation has been widely studied, these enzymes are not relevant for simulation of membrane water treatment conditions due to the high enzyme dosage

applied and their unstable property for denaturation. For example, myeloperoxidase is one of the well-studied enzymes that functions as CNM degradation agent, but it mainly exists in human neutrophil granulocytes (a subtype of white blood cells). Also, chemical cleaning agents can easily inactivate the enzymes and terminate the degradation process. Moreover, certain enzymes might be able to degrade only a certain type of CNM. For instance, Modugno et al. showed that only a subset of the MWCNTs were partially degraded by horseradish peroxidase and that all double-wall CNTs were resistant to biodegradation⁴².

In addition to individual enzyme catalyzed degradation, microorganisms have been shown to degrade CNMs due to their ability to produce extracellular matrix and form complex communities, which can protect the enzymes from inactivation. Microorganisms forming biofilm is the primary cause for membrane biofouling. *Sphingomonas spp.* was chosen for this study because it has been identified at the base of the biofilm in contact with the membrane^{9, 46, 47, 56} and has the ability to degrade PAHs and possesses the relevant enzyme that could catalyze the degradation of CNMs⁷⁸. For example, Liu et al. reported a naphthalene-degrading bacterium that has the ability to degrade GO and relative materials²⁶, and although they did not identify the bacterium species, they suggested that the bacteria might use a metabolic pathway similar to that of naphthalene to degrade graphitic materials. This study further interpreted the mechanism and indicated that the degradation is caused by bacterial attack (removal of electrons) on the defect sites and edges of the material. Interestingly, a similar metabolic pathway might also exist in the key bacteria that causes membrane fouling in desalination condition. Balkwill et al. isolated several *Sphingomonas* from environment and proved that those species can use naphthalene and other aromatic compounds as their sole carbon source⁴³. Similarly, *Sphingomonas* strains that can degrade certain PAHs, such as naphthalene, phenanthrene, and fluorene, were separated from a

petroleum-contaminated site by Zhou et al⁴⁵. The above studies suggest that *Sphingomonas* with the ability to degrade polycyclic compounds might also be able to degrade CNMs. Although additional experiments are required to verify this hypothesis, *Sphingomonas spp.* demonstrates great potential as a degradation agent for this study.

2.5.3.3 *Sphingomonas aromaticivorans*

Sphingomonas aromaticivorans, also known as *Novosphingobium aromaticivorans* (SMCC F199; DSM 12444), is a Gram-negative, rod-shaped, strictly aerobic bacteria belonging to the *Sphingomonadaceae* family. This species was first isolated from terrestrial-subsurface sediments and was identified by Balkwill et al⁴³. *N. aromaticivorans* demonstrate a strong ability to degrade a various range of aromatic compounds, such as benzoate, p-cresol, and m-xylene. More specifically, *N. aromaticivorans* can digest naphthalene and uses many PAHs as the sole carbon source, which shares a similar structure with CNT. Based on the studies mentioned in the previous section, *N. aromaticivorans* has great potential to degrade CNM and is therefore considered as the strain best suited to the experimental approach.

N. aromaticivorans is the species that is likely to be present on RO membranes, and it remains pertinent to the application in this study. In real water treatment situations, although the genre *Sphingomonas* has been identified as the primary biological agent that causes biofouling, no consensus exists as to which exact species plays more of a role than others. Indeed, the compositions of biofilm in each water treatment plant vary due to the diversity of conditions, such as temperature, salinity, pH, existing microorganism species in inflow water, etc. Even *Sphingomonas spp.* have been found in many different treatment facilities, and their specific species can vary. However, the *Sphingomonas spp.* that appears on a RO membrane might contain several species because they are widely dispersed in nature and have nearly 100 identified species.

For instance, a molecular characterization study of the bacterial communities in an RO plant reported 11 *Sphingomonas* strains based on 16S RNA amplification⁴⁷. Therefore, a species that is ubiquitous in the environment and most likely has the ability to degrade CNM is the best option for use as a model. In addition, the designed system can be scaled up to involve other relevant biofilm-forming bacteria from environmental samples in the future.

2.6 Possible CNM Degradation Products

Although the specific degradation pathways of CNMs are unknown, potentially hazardous intermediates and degradation products can be generated in the process. Figure 1 summarizes the chemical structure of DPs that were empirically observed from biologically related or oxidative degradation of CNMs. Those compounds share a similar structure with PAH DPs, which are generated by organisms through enzyme-catalyzed oxidation and ring cleavage pathways⁷⁹. The toxicity of PAHs and their transformation products depends strongly on structure (specific regions, functional groups, and carbon atom positions)³³. For example, dihydrodiols, one of the toxic metabolites of PAH, and other DPs that share a similar structure have been reported as intrinsically toxic to organisms in different degrees⁷⁹. Therefore, the similarity of the DPs between CNM and PAH suggests that those two materials might share the same or similar degradation pathways. In addition, because both CNMs and PAHs are composed of multiple aromatic rings, it is possible that the mechanisms of degradation have certain characteristics in common in the early steps.

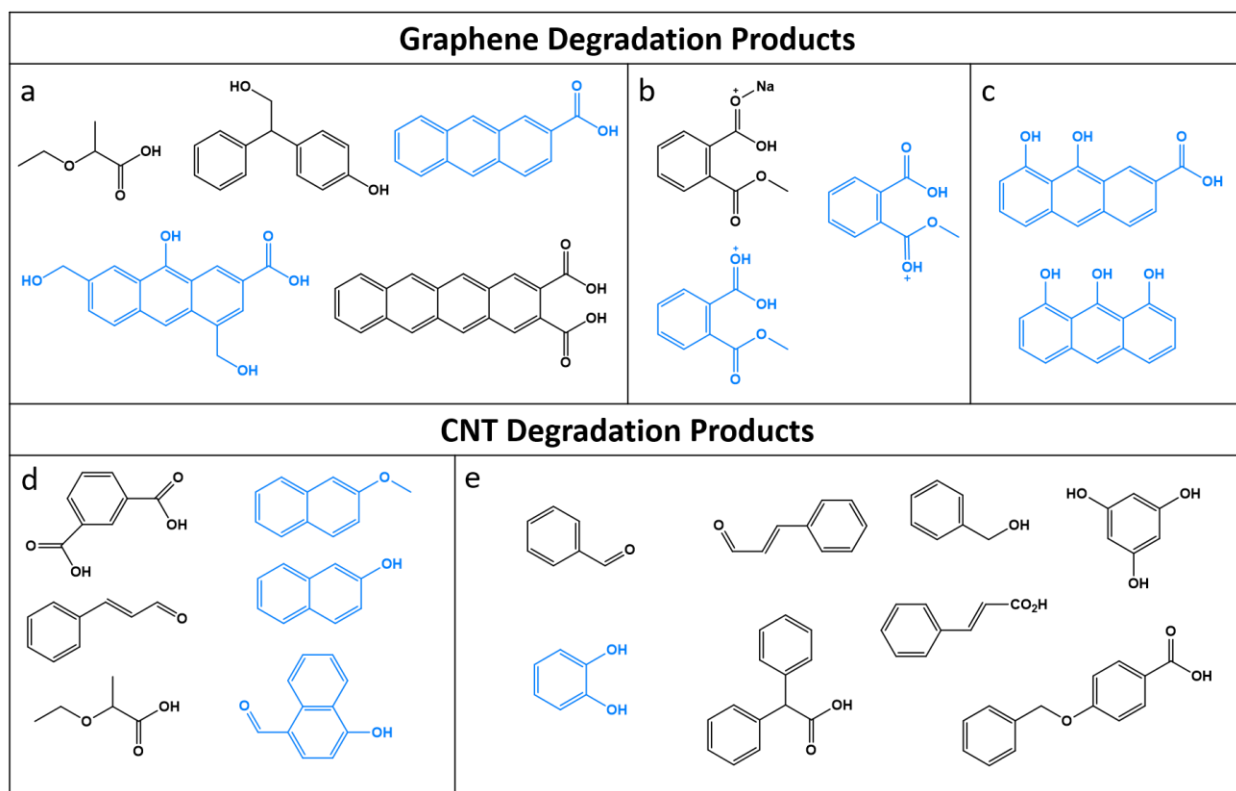


Figure 1 CNM DPs reported by literatures. a) Feng et al. (2015)³⁵; b) Bai et al. (2014)³⁶; c) Hou et al. (2015)⁷⁶; d) Zhang et al. (2013)²³; e) Allen et al. (2009)³⁴. Suspected human carcinogens upstream of the degradation process are marked in blue.

Given the potential for human exposure to harmful DPs from CNM-enabled membranes via recycled water, a comprehensive understanding of their formation and behavior under water treatment conditions is needed.

2.7 Carbon Nanomaterial Degradation Product Separation and Identification

2.7.1 Degradation product identification

Ultraviolet visible spectroscopy (UV-Vis), Raman spectroscopy, Gas chromatography-mass spectrometry (GC-MS), HPLC, Liquid chromatography-mass spectrometry (LC-MS) are commonly used as DP identification methods^{23, 29, 34, 35, 71, 76, 80}. UV-Vis is featured in organic compound analysis, especially for compounds with a high degree of conjugation. Because PAHs and their derivatives are mostly composed of aromatic rings containing a large amount of C=C bonds, their electron conjugation system can be easily detected by UV-Vis. In addition, UV-Vis can be used to quantify the C=C double carbon bonds after the degradation process. By comparing the amount of difference in C=C bonds before and after degradation, information such as degradation rate and mechanism can be obtained.

In addition to UV-Vis, Raman spectroscopy is another technology that is frequently applied to evaluate CNM defects after the reaction. For study of CNM degradation, carbon materials can be sampled at the beginning of or during the experiment and recycled in the end. The changes in the D/G bands of Raman spectra supply information on the percentage of CNM that has been degraded. The G band represents all sp^2 hybridized carbon materials, and the D band is a defect activated band in sp^2 hybridized carbon materials. Based on the ratio of D and G bands, the defect densities can be measured, which can server as an index of CNM degradation. By association with time, the degradation rate can also be obtained. Therefore, this technology is sufficient for revealing the interaction between the material and microorganism.

As a complementary method to Raman spectroscopy, X-ray photoelectron spectroscopy (XPS) measures the surface chemistry, especially the functional group changes of initial and post-exposure CNMs. Compiled with the DP structure, surface chemistry, and total petroleum hydrocarbon might suggest a degradation pathway.

Moreover, HPLC can be implemented to evaluate the variety of DPs present within aqueous media. HPLC uses a column and eluent to separate each compound via differences in molecular polarities. For the type of column (18C reversed-phase) applied in this study, compounds with strong polarities emerge quickly from the column, which indicates that the retention time is short. In contrast, compounds that are more hydrophobic emerge later, demonstrating a longer retention time. However, polarity can be affected by several factors, including the mobile phase (eluent), column type, column temperature, pump speed, and sample volume. Although HPLC has been used as the primary method in this study, it should be noticed that it has many limitations. For instance, HPLC can qualitatively suggest DP formation through the emergence of peaks over time at different retention times but cannot identify the structure associated with those peaks. HPLC also can provide a fast and accurate assay of complex mixtures, but its result can be easily affected by experimental error and impurities. Still, HPLC remain a commonly used method in relevant CNM degradation studies and supplies qualitative analysis for DP formation.

LC-MS and GC-MS are efficient methods for DP identification. For CNM degradation, many studies use dichloromethane or ethyl acetate to extract the potential degradation intermediates. The solution is further concentrated and re-established in methanol before it is injected into the LC unit^{23, 34, 35}. However, it has also been suggested that particles with low boiling point (smaller than about 60°C) could be lost while concentrating the extract. To identify DPs with

low boiling point, GC-MS is recommended by one of the authors²³. At the same time, Feng et al.³⁵ propose that sampling the headspace of each sample for CO₂ could be an effective alternative approach to determining the degradation rate. Furthermore, if peak overlaps are found in LC-MS, Liquid chromatography with tandem mass spectrometry (LC-MS/MS) offers better resolution.

In addition, total petroleum hydrocarbon (TPH) analysis, Fourier transform infrared (FT-IR) spectroscopy, and electron microscopy are applied as supplementary methods in some of the studies to supply extra information on the structures of degradation intermediates^{26, 45, 46, 81}.

2.7.2 Degradation product isolation

HPLC and LC/GC-MS are the major techniques selected for DP identification. However, the methods for sample pretreatment are different across studies. In Feng et al.'s research³⁵, an aqueous-phase reaction mixture was sampled and sequentially extracted three times using dichloromethane; the combined extracted solutions were dried it with a rotary evaporator³⁵ prior to HPLC and LC-MS/MS analysis. Similarly, Zhang et al.²³ applied LC-MS/MS for degradation product identification, but the DPs were extracted with both ethyl acetate and dichloromethane, and the extracts were dried with nitrogen gas to prevent low-MW DP loss during the concentration process. In the research conducted by Allen et al.³⁴, GC-MS, HPLC and LC-MS were applied for possible intermediate product detection; uniquely the sample was acidified with hydrochloric acid and DPs were not extracted with organic solvent.

Although the various DP identification methods mentioned in relevant studies are shown to be functional under their respective conditions, these methods could be affected by experimental variables such as type of nanomaterial, culture broth, microorganisms used, and HPLC settings.

Thus, a comprehensive study of the pretreatment or DP separation methods under specific conditions is required.

3.0 Materials and Methods

3.1 Materials

3.1.1 Single Wall Carbon Nanotubes (SWCNTs)

SWCNTs with 1-2 nm diameter, 1-3 μm length, and 95% purity were selected as the primary CNM for this study. The SWCNTs (GCS121) were purchased from Carbon Nano Tubes Plus (101 N Commerce St, Madisonville, TX 77864) and used as received. They were produced by chemical vapor deposition (CVD). The dimensions of the SWCNTs were within the specified range used in many membrane modification studies. High purity (reported as $> 95\%$) was chosen to eliminate errors caused by impurities. Due to the nature of manufactural process, trace metals can be found as catalyst residual. The analyzation result provided by the manufacturer indicates a generally low ($<1\%$) level of impurities based on energy dispersive X-ray spectroscopy, but with the exception of Co ($<3\%$).

This particular CNM was selected for a number of reasons. First, the SWCNT is a well-studied CNM that is applied for membrane performance enhancement. A notably large number of studies have been conducted that show the improved performance of SWCNT-enabled membranes, including better mechanical properties²², high contaminant rejection⁸², high water permeation¹⁶, and antimicrobial abilities^{63, 83}. The related research is rapidly increasing each year, suggesting the feasibility of SWCNT as a membrane enhancing material. Second, SWCNT has morphological consistency. Although both graphene and SWCNT are one-atom-thick carbon allotropes (the SWCNT is similar to rolled graphene), the dimension of graphene vary in same

sample. Similarly, although MWCNT share the same cylindrical structure, its wall might vary from double to multiple layer in same sample. Choosing SWCNT as the model CNM determines its transmission under water treatment conditions, and the results are also informative for relevant studies on MWCNT and graphene.

3.1.1.1 Characterization

The SWCNTs used in this study were characterized using transmission electron microscopy (TEM). SWCNTs were suspended in purified water to achieve a concentration of 100 µg/ml. The suspension was dropped onto a copper grid for analysis. The images were collected with FEI Image Corrected Titan 60/300 S/TEM microscope at Oak Ridge National Laboratory.

Thermal gravity analysis (TGA Q5000, TA Instruments, 159 Lukens Drive, New Castle, DE) was conducted to evaluate the carbon purity. Nitrogen was used as a balance gas, and compressed air was used as the sample gas, with flows of 10 ml/min and 25 ml/min, respectively.

3.1.2 *Sphingomonas aromaticivorans*

The type strain of *N. aromaticivorans* was purchased from American Type Culture Collection (ATCC® 700278™, 10801 University Boulevard, Manassas, VA). The bacteria were activated from the lyophilized pellet by culturing with tryptic soy broth, and aseptically transferred into a culture tube. The well-mixed suspension was used to inoculate other broth, slants, and plates. To preserve the bacteria, a sterile 50% glycerol solution was added to broth cultured overnight at a ratio of 1:1. The culture vial was stored at -80 °C.

3.1.2.1 Culture Conditions

According to the product sheet for *Novosphingobium aromaticivorans* from ATCC, this species requires aerobic conditions for growth. The optimal growth temperature is 30°C. In this study, two types of culture medium were used, tryptic soy (TS) broth/agar and mineral salt medium (MSM).

TS Broth

Bacto™ Tryptic Soy Broth (lot 6357914, BD, 1 Becton Drive Franklin Lakes, NJ) and Tryptic Soy Agar (lot 3086270, BD, 1 Becton Drive Franklin Lakes, NJ) are used in bacteria enrichment. TS broth or agar contains NaCl, dextrose, K₂HPO₄, tryptone and soytone. Dextrose is the main carbon source within the medium, and tryptone and soytone are the sources of nitrogen. Tryptone is a specially formulated enzymatic digest of casein, which is reported to contain various amino acids: Ala 2.8%, Arg 3.2%, Asp 6.7%, Cys 0.3%, Glu 18%, Gly 1.8%, His 2.4%, Ile 4.7%, Leu 7.7%, Lys 7.0%, Met 2.5%, Phe 4.1%, Pro 9.1%, Ser 5.1%, Thr 3.9%, Trp 1.0%, Tyr 1.3%, and Val 6.0%. For inorganic components, tryptone contains Ca, K, Mg, and Na. Soytone contains many types of amino nitrogen and mineral salts, such as NaCl, Ca, Fe, Mg, K, Na, Cl, and S. Although tryptone and soytone serve as nitrogen sources, *Sphingomonas* is able to grow on each of them due to the presence of carbon in those ingredients and the ability of *Sphingomonas* to take nutrients from oligotrophic conditions. In addition, with respect to the application of TS broth/agar with CNM and PAH, no study was found that suggested any reaction between the broth/agar and those materials.

Mineral Salt Medium (MSM)

MSM is an adjusted buffer containing only inorganic salts. MSM is widely used in studies of bacteria metabolism, nutritional requirements, and strain screening. The recipe for MSM is shown in Table 5. In this study, MSM was used to determine the PAH- and CNM-degrading capacity of *N. aromaticivorans*.

Table 5 Recipe for mineral salt medium. The final pH is 7.0-7.2.

		Trace elements	
Name	concentration (/L)	Name	concentration (/L)
K ₂ PO ₄	2 g	FeSO ₄ 7H ₂ O	2.00 g
KH ₂ PO ₄	0.5 g	MnSO ₄ H ₂ O	2.00 g
NaCl	0.5 g	Na ₂ MoO ₄ 2H ₂ O	0.44 g
NH ₄ Cl	0.5 g	H ₃ BO ₄	0.20 g
MgSO ₄	0.2 g	CuSO ₄ 5H ₂ O	0.40 g
CaCl ₂	10 mg	ZnSO ₄	0.50 g
		NH ₄ VO ₃	0.20 g
		CoCl ₂ 6H ₂ O	0.50 g
		NiCl ₂ 6H ₂ O	0.20 g

MSM benefits the study in multiple ways. First, the ingredients are much simpler than those of TS broth, which avoids complicated analytical results caused by enzymatic digested peptides. Second, carbon source can be limited to one or several specific compounds, such as PAH and SWCNT, which aids in understanding whether those aromatic compounds can be metabolized without extra carbon sources. However, based on our experimental results, the ability of *N.*

aromaticivorans to develop a biofilm is poor with MSM, which suggests that MSM might not be an appropriate medium for biofilm growth.

3.1.2.2 Viability Assay

The OD₆₀₀ method was chosen in this study for evaluation of bacteria growth. In broth medium, the solution turns turbid, and the viscosity increases with bacterial growth. The bacteria suspension was measured with a microplate reader (Synergy HT, BioTek Instruments Inc., 100 Tigan St, Winooski, VT). For agar plates, the color and shape of the colony is used to determine the growth of *Sphingomonas*, because one of its salient features is that it produces yellow- or off-white-pigmented colonies.

Although OD₆₀₀ is considered sufficient for current study, comprehensive viability assay can be conducted using MTX, XTT, and WST-1⁸⁴. However, a commonly used viability assay, MTT, cannot be applied on the sample containing CNT. Knirsh's group found that SWCNTs are able to interact with commonly used tetrazolium salts, such as MTT⁸⁵. His paper indicates that insoluble MTT-formazan crystals can lumped with nanotube and clumped them together, which resulting in an incorrect result of the assay. To assess the cytotoxicity of SWCNTs, propidium iodide (PI) and 4'-6-diamidino-2-phenylindole (DAPI) are the most widely used options^{63, 86, 87}.

3.1.2.3 Growth Curve

The *Sphingomonas* growth curve was studied via a microplate reader to determine the time required for a newly inoculated culture to achieve the stationary phase. This analysis also confirmed whether SWCNTs adversely affect their growth.

Sphingomonas bacteria were picked from the TS agar plate and inoculated in 10 ml TS broth under sterile conditions. The culture tube was incubated overnight at 30°C. At first, SWCNTs

were added to the growth media to achieve 1 g/L, 0.1 g/L and 0.01 g/L dosages based on the stock concentration used in previous studies of SWCNT-enabled membranes. The grown culture was diluted 10-fold in TS broth and added to culture vials with and without 0.1 g/L SWCNT. Each sample had three replications. The culture plate was placed in the microplate reader at 30°C for 32 hours, and the absorbance wavelength was set to 600 nm. The absorbance of the TS broth was measured as a blank.

3.1.3 Membrane Cleaning Agents

NaOH, EDTA, and SDS are selected as representative cleaning agents and are used for removing organic foulants from membranes (see above).

3.1.3.1 NaOH

NaOH is a commonly used membrane agent due to its ability to hydrolyze and dissolve organic foulants. ACS certified-grade NaOH (lot 141102, Fisher Scientific, Assay 99.1%) was used to make an alkali solution with pH = 11.

3.1.3.2 EDTA

EDTA is a metal chelating agent and has six positions to form coordinate bond with fouling particles. The complexes of EDTA and fouling particles are expected to be separated from the membrane surface. ACS certified-grade EDTA (lot 893367, Fisher Scientific, Assay 99.8%) was used in this study. 1 M NaOH was used to adjust the pH to form Na₂-EDTA.

3.1.3.3 SDS

SDS (lot 269216, Fluka™, Assay > 85%) is an anionic surfactant containing both hydrophilic and hydrophobic groups. This surfactant can solubilize large hydrophobic molecules by forming micelles around them.

3.1.4 HPLC

An Agilent 1200 series HPLC system equipped with a 1260 refractive index detector and a 1314B VWD detector was used for material analysis and possible DP detection. All HPLC spectra were analyzed with Agilent ChemStation software. The specific parameters applied for HPLC are given as follows.

3.1.4.1 Column

The 150 mm and 250 mm reverse-phase C18 columns were used in this study. The shorter column was applied for PAH standard spectrum analysis, whereas the longer column was used in all other measurements. Switching to a longer column can achieve better separation of DP and a higher resolution spectrum. In addition, due to the nature of the reverse-phase C18 column, hydrophilic compounds have a shorter retention time, whereas hydrophobic compounds have a longer retention time.

3.1.4.2 Mobile Phase (Eluent)

Based on a literature review, an isocratic eluent composed of 70% deionized water and 30% acetonitrile (Optima®, Lot 185397, Fisher Chemical) is the mobile phase most commonly used in detection of CNM DPs. However, in a few studies, a mobile phase of 90% water and 10%

acetonitrile was also chosen. The mobile phase is the liquid that flows through the stationary phase (the column). The composition of the mobile phase affects the final HPLC spectrum obtained because the strength of bonding between the mobile phase and the compounds depends on the gradient.

The two mobile phases were compared using TS broth. Because the result indicated no difference between those two methods, the mobile phase with 70% water and 30% acetonitrile was selected for this study.

3.1.4.3 Wavelength

A wavelength of 230 nm was set for VWD. Most PAHs have a high absorption value and are better resolved at this wavelength. This value is also applied in other related studies.

3.1.4.4 Other Parameters

The column temperature was set to 30°C. The pump speed for the mobile phase was 1 ml/min, and the injection volume for each sample was 20 µl.

3.1.5 Model PAHs

PAHs are used in evaluation of DP separation and detection methods and in verification of the ability of bacteria to digest aromatic hydrocarbons. In this section, the characteristics of the model PAHs are studied.

Naphthalene (98%, Lot 031997, Aldrich Chemical Company, Inc.), fluoranthene (98%, Lot 1730JH, Aldrich Chemical Company, Inc.) and anthracene (99.9+%, gold label, Aldrich Chemical Company, Inc.) were selected as model PAHs because of their potential adverse health

implications. Fluorene (98%, lot 5811HE, Aldrich Chemical Company, Inc.) was also selected because its five-carbon-ring structure might serve as a defect site. Because the other components of fluorene are similar to fluoranthene, the influence of the defect site can be studied by comparing the results from these two compounds.

3.1.5.1 Standard HPLC Spectrum for PAHs

Naphthalene, fluoranthene, fluorene, and anthracene were diluted in HPLC-grade methanol (A454-1, LOT179317, Optima®, Fisher Chemical) to a concentration of 1 g/L. The solutions were directly analyzed by HPLC, and the results are shown in section 4.2.

3.1.6 DP Isolation

Dichloromethane and ethyl acetate are the most commonly used organic solvents in CNM DP extraction^{23, 34, 35, 71, 78}. Both are polar solvents and are miscible with a variety of other organic solvents. However, no appreciable difference exists between the two solvents. Certain researchers chose one of them^{35, 78}, and others applied both^{23, 34}. In this section, DP extraction of dichloromethane and ethyl acetate are compared from the perspective of HPLC analysis, and their standard HPLC spectra are also determined to exclude any error peaks caused by residual solvent.

3.1.6.1 Dichloromethane

HPLC-grade dichloromethane (lot SHBJ0856, Sigma-Aldrich®, Assay > 99.8%) was used in this study, and its HPLC spectrum is shown in Figure 2(a).

3.1.6.2 Ethyl Acetate

HPLC-grade ethyl acetate with a purity of 99.9% was purchased from Fisher Chemical (E196SK, Lot 181285, Optima®). The standard spectrum of ethyl acetate is shown in Figure 2(b).

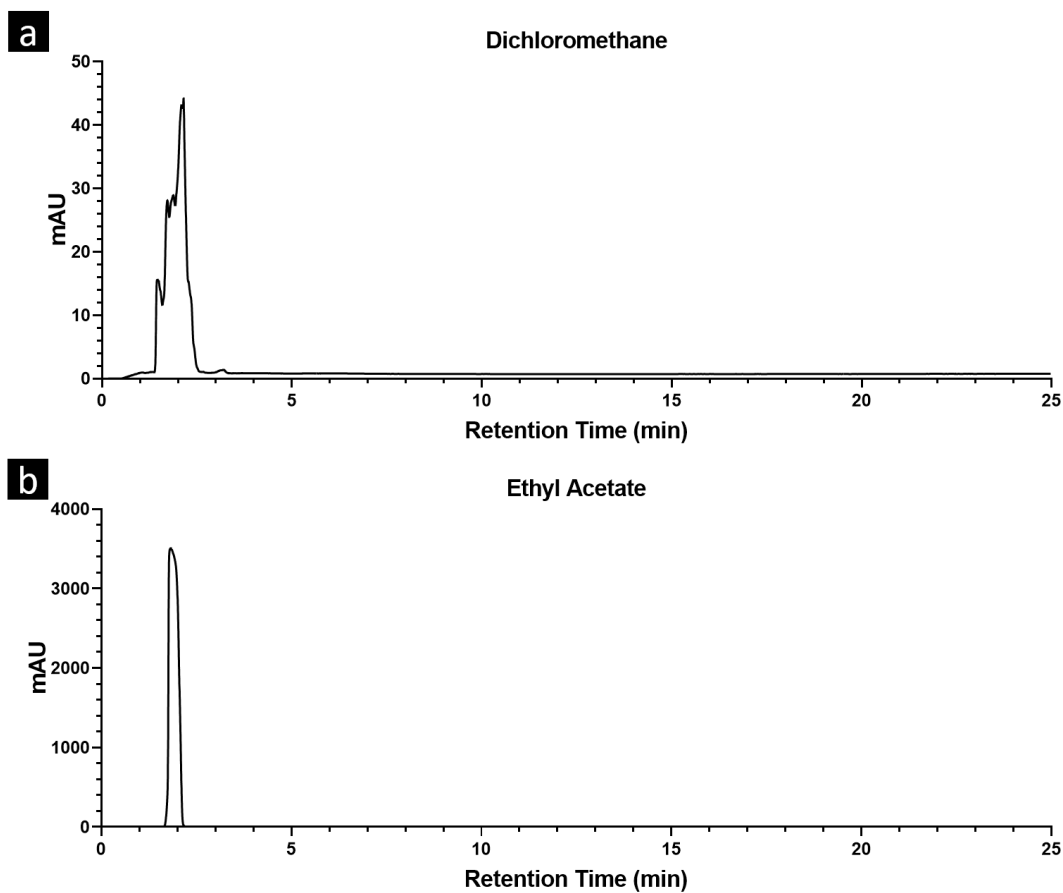


Figure 2 Standard HPLC spectra of dichloromethane and ethyl acetate.

As can be seen in Figure 2, ethyl acetate has a simpler peak than dichloromethane under the specific HPLC condition used in this study. The scattered HPLC spectrums of dichloromethane

probably caused by stabilizers contained in the reagent, such as amylene, based on the analytical results on the label.

The single peak of ethyl acetate benefits the subsequent HPLC analysis because it is less likely to overlap with the peak from DPs. If any residual remains in the sample, it is easy to identify. Furthermore, ethyl acetate is known as one of the “green solvents”, with minor health and environmental impacts⁸⁸. Therefore, ethyl acetate is suitable for this study.

3.2 Methods

3.2.1 DP Isolation Methods

3.2.1.1 Extraction

Samples of culture media were extracted before and after the experiments using ethyl acetate at a ratio of 1:1. The mixture was vortexed with a maximum rotation speed (3000 rpm) for 30 seconds and centrifuged at 12000 rpm for 10 minutes to remove any insoluble particles. The speed of centrifugation is determined by Stoke’ law, which indicates that 12000 rpm (relative centrifugal force > 700) is capable to separate bacteria, cells, precipitates, and macromolecules. The supernatant (extracts) was transferred to another container, concentrated with a rotary evaporator, and analyzed by HPLC.

3.2.1.2 Direct Injection

Considering that possible DPs might be lost in the extraction and concentration process, an HPLC assay with direct sample injection was also applied to verify the results of other method.

Since this method does not involve concentration process, the HPLC peak intensity of PDs detected by this method are expected to be lower. The culture medium was first centrifuged at 12000 rpm for 10 minutes to remove all bacteria and CNMs, and the supernatant was directly injected into the HPLC for analysis.

3.2.1.3 DP Concentration

To obtain distinct results of possible DPs in small amount, the extracted samples were concentrated by rotary evaporation in a 60°C water bath at rotary speed of 35 rpm and pressure of 350 mbar. The extracted solution was dried, and the residue was subsequently dissolved in 1 ml of methanol. The reconstituted solution was placed into 1.5 ml centrifuge tubes and centrifuged at 12000 rpm for 10 minutes. It should be noted that volatile DPs, especially those with low boiling points, cannot be detected by this method. The reason for using methanol is because it can dissolve nonpolar PAH compounds and their polar derivatives at the same time.

3.2.2 Biofilm Culture

N. aromaticivorans biofilm was grown on well plates based on the following protocols. First, 10 ml TS broth in 15 ml tubes was prepared for overnight culture. One *N. aromaticivorans* colony was picked up with a sterilized loop or wooden sticks from TS agar plates and was inoculated in 10 ml TS broth under sterile conditions. The culture tubes were placed in a test tube rack on a shaker inside a 30°C incubator to grow the bacteria for approximately 24 hours. The shaker speed was set at 150 rpm. The overnight culture was diluted 100-fold in fresh TS broth. The newly made cultures were transferred to well plates and covered with a plate lid under sterile

conditions. The plate was placed in a 30°C incubator and shaken gently at 150 rpm. Culture media were carefully changed every 24 hours without breaking or removing the biofilm from the well.

Usually, the biofilm continues to increase its sizes during a period of 3 days. Subsequently, the biofilm size and morphology stabilize, at which time the biofilm is considered “mature”. The progress of each day is shown in Figure 3.

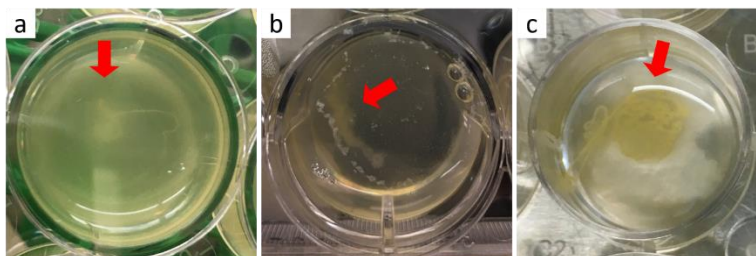


Figure 3 *N. aromaticivorans* biofilm at a) 1st day, b) 2nd day, and c) 3rd day. The biofilm formed in each day is marked by arrows. (the color is affected by the light when taking the picture.)

The broth was slowly removed from the upper layer of the well with a 1 ml pipette. The biofilm on the well bottom has an amorphous and loose shape and is easily distinguished by its shape and deeper color. During removal of the broth from the well, no biofilm should be collected or disrupted.

3.2.3 PAH and SWCNT Degradation

To determine the SWCNT degradation capability and the metabolism pattern of *N. aromaticivorans*, various approaches were applied to limit the carbon source available in the culture media.

3.2.3.1 Inorganic Nitrogen Source

According to the previous section on culture conditions, TS broth is composed of soytone, tryptone, dextrose, NaCl and K₂HPO₄. Dextrose is the major carbon source, soytone and tryptone are nitrogen sources, and NaCl and K₂HPO₄ supply mineral elements and balance the osmotic pressure and pH. Our pre-experiment indicates that *N. aromaticivorans* is able to grow on soytone or tryptone in the presence of NaCl and K₂HPO₄ due to their ingredients, mostly peptides, which contain carbon. This result shows that an alternative inorganic nitrogen source is required to limit the carbon source to the target material.

Based on this result, nitrate, nitrite and ammonium were tested as inorganic nitrogen sources. The amount of salts is calculated according to the nitrogen ratio in both tryptone and soytone. To ensure that the carbon source can be used by the microorganisms, dextrose was added at the same time. All of the media were prepared in the form of agar plates. The overnight culture was diluted 100-fold with 0.9% saline solution and inoculated on each plate. Three replicates were prepared for each sample.

3.2.3.2 PAH and SWCNT Degradation with Restricted Carbon Source

This experiment tested the ability of *N. aromaticivorans* to degrade aromatic compounds. The representative PAHs (fluorene, naphthalene, anthracene and pyrene) and pristine SWCNTs

were applied as the only carbon source for bacteria growth. By conducting this experiment, a direct result for SWCNT degradation ability can be obtained. This experiment also supplies additional information related to the bacteria metabolism on aromatic compounds.

MSM, an inorganic salt buffer that contains various elements but any carbon source, was used in this experiment. The ingredients of MSM are shown in Table 5. MSM agar plates were prepared by adding 0.4 ml of each trace element solution (from 20 ml stock) into 400 ml of MS solution. An amount of 6 g of agar was also added into the mixture. Four PAHs, SWCNTs, and dextrose were prepared at a concentration of 1 g/L in acetone and sonicated for 5 minutes prior to mixing with the autoclaved MSM agar. The mixture was separated into seven Erlenmeyer flasks, with 45 ml for each flask. Acetone-only MSM agar and TS agar were also prepared as controls. Table 6 shows the carbon source for each sample.

Table 6 Ingredients of each sample for PAH and SWCNT degradation experiment.

Sample No.	Medium	Carbon Source	Notes
A	MSM	Dextrose	Control
B	MSM	SWCNT	CNM
C	MSM	Fluorene	PAH
D	MSM	Naphthalene	PAH
E	MSM	Fluoranthene	PAH
F	MSM	Pyrene	PAH
G	MSM	N / A	Eliminate affects caused by acetone.
H	TS Broth	N / A	Confirm bacteria growth normally.

Aliquots of 10 ml of each autoclaved media were placed on Petri dishes. For each sample, 3 replications were examined. The bacteria were diluted 100-fold with 10 ml of 0.9% saline solution. After the plate became solid, 100 μ l of diluted bacteria solution were inoculated on each plate. All samples were cultured aerobically at 30°C.

3.2.4 SWCNT Degradation Experiment

In this experiment, SWCNT and TS broth were used to determine whether they can be biologically degraded through cometabolism. *N. aromaticivorans* were cultured in 500 ml Erlenmeyer flask with 250 ml of media. The TS broth was exchanged every 24 hours for 3 days to develop biofilms. After the biofilm was formed, 0.1 g/L SWCNT was prepared by mixing the nanomaterial with autoclaved TS broth and applying it on the biofilm. A bacteria-only culture was prepared at the same time as a control. All broth change operations were performed under sterile conditions. The flasks were placed in a 30°C incubator and shaken at 150 rpm. The possible DPs were sampled using the extraction and concentration method and analyzed by HPLC.

3.2.5 Degradation of SWCNTs Treated by Membrane Cleaning Agents

Chemical cleaning reagents involved in membrane cleaning pose the risk of promoting CNM degradation. In this subsection, NaOH, ethylenediaminetetraacetate (EDTA) and sodium dodecyl sulfate (SDS) were used as chemical cleaning agents because of their popularity in commercial cleaning products for organic-fouled membranes. In a study conducted by Ang et al.⁴⁹, the efficiencies of sodium hydroxide, EDTA and SDS under different conditions were examined.

Based on this result, the most efficient condition was selected, e.g., pH=11 and a temperature of 40°C.

In the experiment, 100 ml of DI water was added into three flasks. The chemical cleaning reagents were added to each flask, and the pH was adjusted with 1 M NaOH. The pH value was confirmed by both pH paper and pH meter. The details of each sample are shown in Table 7.

Table 7 pH value and mass of chemical cleaning reagents applied on SWCNTs.

Sample	Reagents	NaOH used for adjusting pH	Final pH	Recycled SWCNT mass
A	NaOH	500 μ l	11.57	43.122 mg
B	EDTA 10mM	4.3 ml	11.38	40.342 mg
C	SDS 10mM	600 μ l	11.16	47.764 mg

After preparing the cleaning solution, 50 mg of SWCNT were placed into each flask and sonicated for 10 mins for better dispersion. SWCNTs were pretreated for 300 minutes based on an assumption of 10 cleaning processes for 30 minutes each time. The pretreated materials were washed with DI water 10 times. After each wash, the materials were isolated by centrifugation (10000 rpm, 5 mins). The recycled SWCNTs were dried in a desiccator overnight and weighed. The SWCNTs were added to an MS agar plate to determine whether the SWCNTs can be used as the only carbon source. The treated SWCNTs were also added to TS broth to determine whether the SWCNTs can be degraded by cometabolism. In addition, the mixture of TS broth and cleaning reagent was analyzed to ensure that they did not react. As a control, cleaning reagents were subjected to the same process without SWCNTs and analyzed by HPLC after extraction and concentration.

For the agar plate, 1 g/L of each pretreated SWCNT and agar were added to MS broth, and 1 g/L of dextrose was used as a control. The dextrose concentration corresponds to a eutrophic condition, in which the bacteria can grow better. After autoclaving all of the mixtures, each mixture was poured into three plates and inoculated with bacteria.

For the TS broth, 0.1 g/L of each pretreated SWCNT was added to TS broth with 3-day-old biofilm. The mixtures were shaken and cultured at 30°C for 7 days. All samples were extracted and concentrated prior to HPLC analysis. The SWCNT concentrations selected in this experiment are based on a literature review and previous experiments.

3.2.6 DP Isolation Methods Development

Because it is already known that various PAHs can be degraded by *N. aromaticivorans*, they are perfect for evaluating DP isolation methods. Two DP isolation methods were developed in this study. The first method is extraction and concentration. By following this method, possible DPs are extracted by organic solvent and concentrated with a rotary evaporator to a final 1 ml solution. The second method is direct injection in which centrifuged samples are directly injected into the HPLC.

Although both methods are theoretically feasible for DP isolation, their effectiveness is unknown without further evaluation. Therefore, this section focuses on verifying the two methods using different approaches. The results are discussed in the next chapter.

3.2.6.1 Preliminary Studies

Extraction and Concentration

Because it is already known that various PAHs can be degraded by *N. aromaticivorans*, they are the perfect candidates for evaluating DP isolation methods. In this experiment, naphthalene, fluoranthene and fluorene were applied on *N. aromaticivorans* biofilm, and their DPs were collected after 7 days of degradation via the extraction and concentration method. The procedures are described below.

Three-day-old *N. aromaticivorans* biofilms were prepared following the previously described procedure. PAH solutions were prepared by dilution in acetone. The solutions were mixed with TS broth to achieve a 1 g/L PAH concentration. A control sample with only acetone and TS broth was prepared as well. All samples were transferred to well plates and cultivated for 7 days at 30°C. The remaining PAH-TS broth mixtures were extracted and concentrated for HPLC analysis. The results were used as the data for day 0.

Direct Injection

By considering the possibility that DPs with a low boiling point might be lost during extraction and concentration, an HPLC assay with direct sample injection was performed to determine the validity of the separation methods. This experiment compared the HPLC spectra of TS broth and PAHs treated by both methods. The PAH standard spectrum measured in previous experiment (Section 3.1.5.1) was used as a reference for determining the parent peaks in the sample.

TS Broth

For the samples with extraction, TS broth was first mixed with the same volume of ethyl acetate. The organic supernatant was separated from the mixture and rotary evaporated to dryness, and an amount of 1 ml of methanol was added. Finally, the re-established solution was injected into the HPLC to obtain the spectrum.

For the samples without extraction, TS broth was first filtered by a 0.22 μm syringe-type PTFE filter to remove any possible solid particles (because directly injecting solid particles into the HPLC can cause irreversible damage, such as a blocked column and pump failure. The chemical compatibility sheet supplied by the filter manufacture does not contain any information indicating that the filter could interreact with the TS broth.) The filtered TS broth was directly injected into the HPLC. The same HPLC configurations were applied for all samples.

PAH Samples

The purpose of this experiment is to determine whether the direct injection method is suitable for separation of nonpolar products such as PAH. Naphthalene, fluorene, and fluoranthene are used to evaluate the effectiveness of the method.

PAHs were dissolved in acetone and mixed with TS broth to achieve a 1 g/L PAH-broth mixture. Although the use of acetone to dissolve PAH is a method commonly used in other studies, precipitation occurs when the PAH-acetone solution was added to water because of the changes in solubility. Therefore, to prevent HPLC damage, the mixture was filtered with a 0.22 μm syringe-type PTFE filter to remove all insoluble particles. Depending on the sensitivity of the HPLC (10 mg/L, determined by using model PAHs), it could be useful to start with lower concentrations that are more soluble. Thus, 0.1 g/L and 0.01 g/L PAH-broth mixture were tested as well. In addition,

PAH dissolution agents (e.g., acetone or methanol) and insoluble particle removal methods (e.g., filtration or high-speed centrifugation) were also investigated.

For the extracted samples, ethyl acetate was added to the filtered mixture. The supernatant was subsequently collected for drying via rotary evaporation. An amount of 1 ml of methanol was added to the dried sample to re-establish the solution. Alternatively, other samples were directly injected into the HPLC.

3.2.6.2 Comprehensive PAH Degradation Experiment

Based on the previous PAH degradation experiment, the experimental procedure was refined. Samples were collected every two days and processed with both separation methods at the same time to obtain continuous DP changes. Fluoranthene and anthracene were used for these experiments.

Fluoranthene

Fluoranthene was selected because its HPLC peak is simpler and more distinct based on the results obtained in previous section (4.2), and thus it is easier to distinguish the peaks caused by DP in subsequent samples. In this experiment, a 6-well culture plate was used to grow biofilm. Acetone-diluted fluoranthene was applied on three-day-old *N. aromaticivorans* biofilms to prepare a 0.01 g/L mixture. Biofilm-only samples were prepared as a control. On day 0, the PAH-TS broth mixture was also treated with two separation methods and analyzed by HPLC. The culture plates were placed in a 30°C incubator and shaken at 150 rpm. Two replicates were prepared for each sample. The methods applied for sampling each well are shown in Figure 4.

At the determined time point, for the extraction and concentration samples, 8 ml was collected from each well and combined into 16 ml. The same volume of ethyl acetate was added

to extract all possible DPs. The mixture was vortexed for 1 minute and centrifuged at 12000 rpm for 10 minutes. The supernatant was transferred to the rotary evaporator to distill all ethyl acetate from the sample. An amount of 1 ml of methanol was added to the residue. The reconstituted solution was centrifuged at 12000 rpm for 10 minutes and analyzed by HPLC.

For the direct injection samples, 0.75 ml was collected from each well and combined into 1.5 ml. The sample was centrifuged as 12000 rpm for 10 minutes to remove bacteria and solid particles. It is possible that 12000 rpm centrifuge can rupture the cells, but organelles, such as ribosomes and polysomes, and macromolecules can be effectively separated. Thus, we assume that all of the insoluble particles, including those affect HPLC results, are removed by centrifuge. The samples were then injected into the HPLC for analysis.

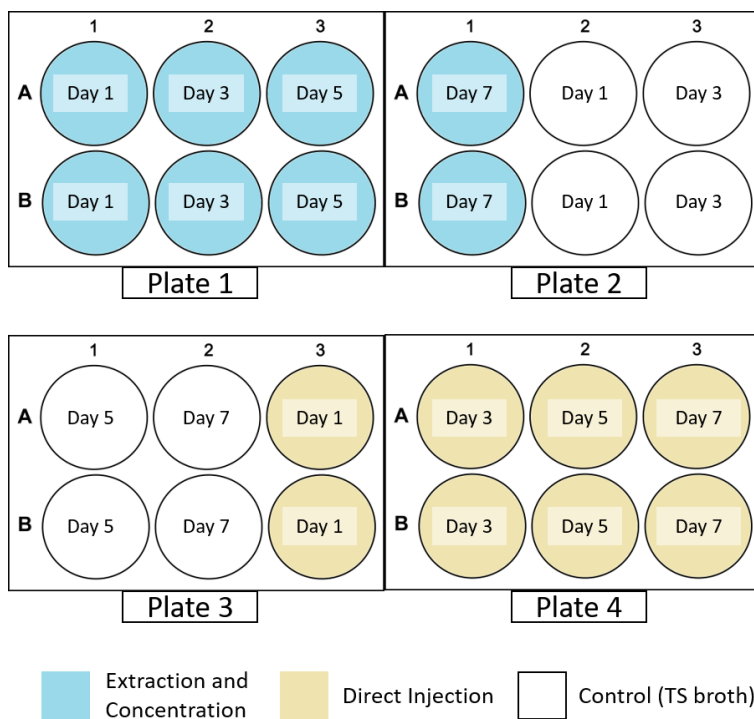


Figure 4 Sampling procedure for fluoranthene degradation experiment.

Anthracene

To verify the separation methods and the aromatic compound degradation capacity of the bacteria, anthracene was used in this experiment. The experimental procedure is same as described in Section 3.4.2.1. However, if nonsignificant or nondetectable degradation is observed on the 7th day, the experiment could be extended up to 11 days. The methods applied for sampling each well are shown in Figure 5. This experiment also separated different culture conditions in different plates to avoid contamination. A solution of 0.01 g/L anthracene in TS broth was also measured at on day 0 of the experiment as a blank sample. This sample supplies information for comparing the differences before and after bacterial degradation.

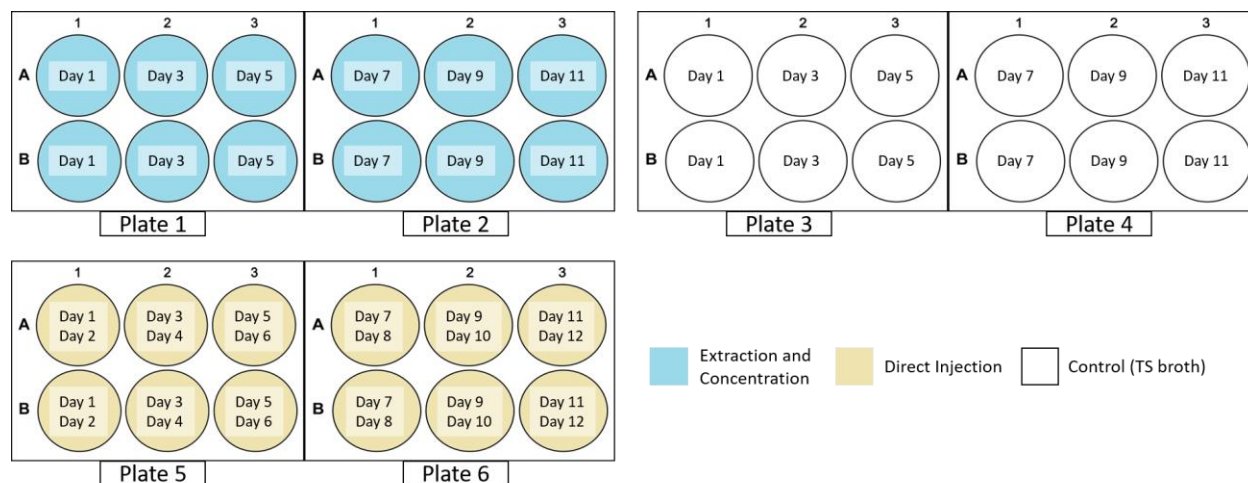


Figure 5 Sampling procedure for anthracene degradation experiment.

4.0 Results and Discussion

4.1 SWCNT Characterization

4.1.1 TEM Images

Figure 6(a) confirms that the SWCNT length is approximately 1-3 μm . Figure 6(b) and Figure 6(c) are magnifications and further confirm that the diameter is approximately 1-2 nm. As shown in Figure 6, individual nanotubes tend to align themselves together into a thinner line, caused by a type of van der Waals forces known as π - π stacking^{14, 40, 50, 89}. Such behavior and their hydrophobicity make SWCNTs difficult to disperse evenly in a water solution.

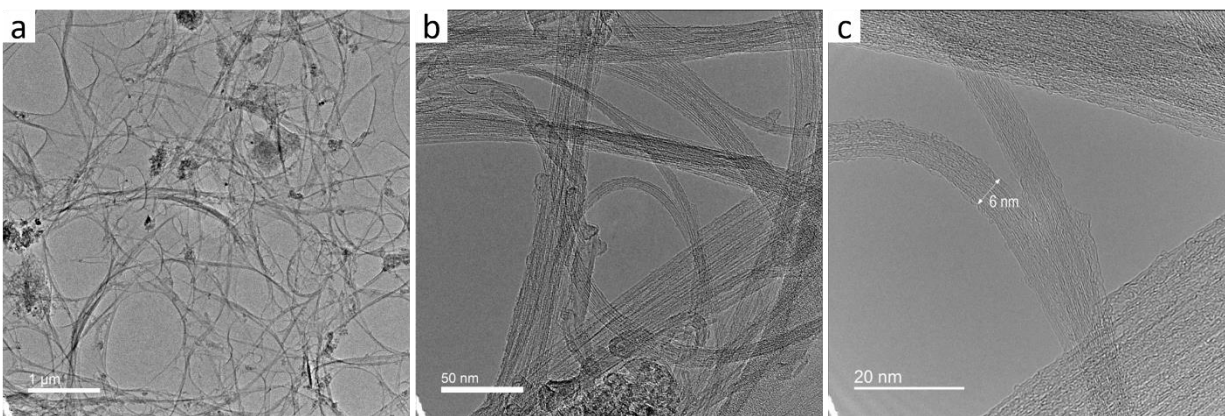


Figure 6 Transmission electron microscopy of SWCNTs used in experiments. The scales are a) 1 μm , b) 50 nm, and c) 20 nm. In c), four SWCNTs are bound together, and their diameter is approximately 1.5 nm.

4.1.2 TGA Curve

Thermalgravimetric analysis was conducted to evaluate the carbon purity. The percentages of mass loss and mass change are shown in Figure 7. The purity of SWCNT was determined to be 97.7% (3.3% residual). Generally, the moisture contained in the material evaporates as the temperature increases, as represented by the derivative of mass change at temperatures below 100°C. Because amorphous carbon impurities burned at lower temperatures than the SWCNT, the smooth mass change curve at relatively low temperature (100-400°C) indicates that amorphous carbon was nearly nonexistent in the sample. In addition, SWCNT began to transition to carbon dioxide at temperatures higher than 480°C, and its ignition point was near 620°C.

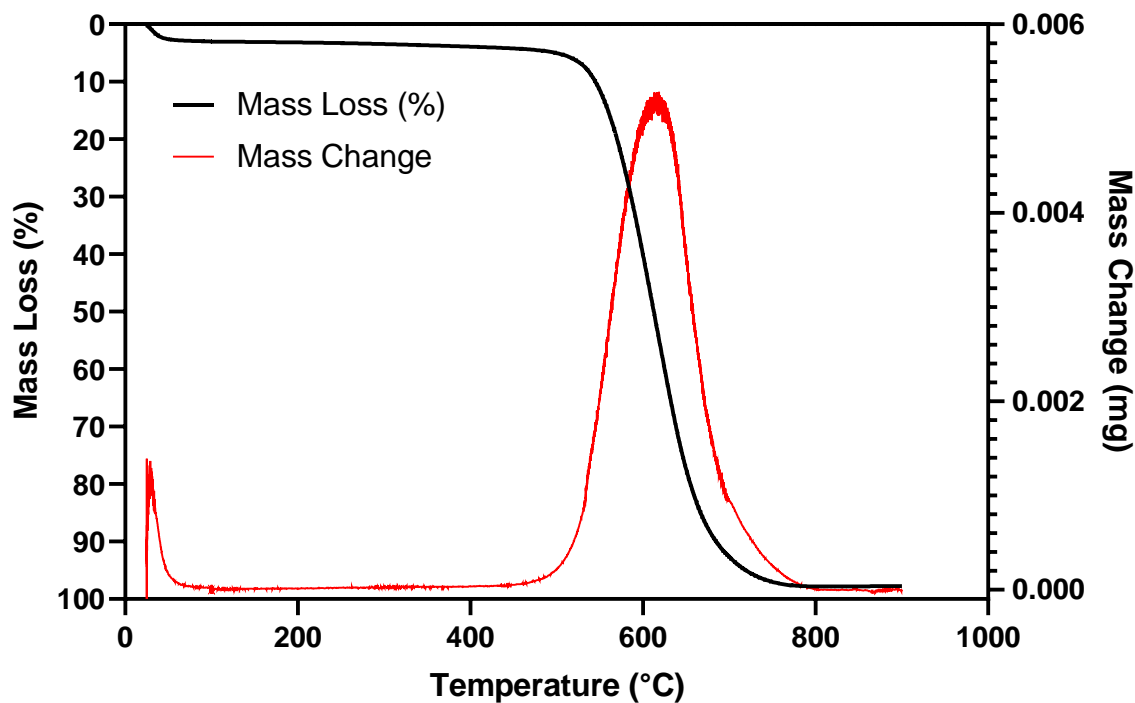


Figure 7 Thermal gravimetric analysis (TGA) graph of the SWCNTs.

4.2 Standard HPLC Spectrum for PAHs

Except for fluorene, a distinct single peak was observed in all other PAHs. The smaller peaks detected in fluorene are probably impurities or its degradation products. The HPLC spectrum of methanol is also shown in the Figure 8 as a control. The retention times of each of the detected peaks are summarized in Table 8.

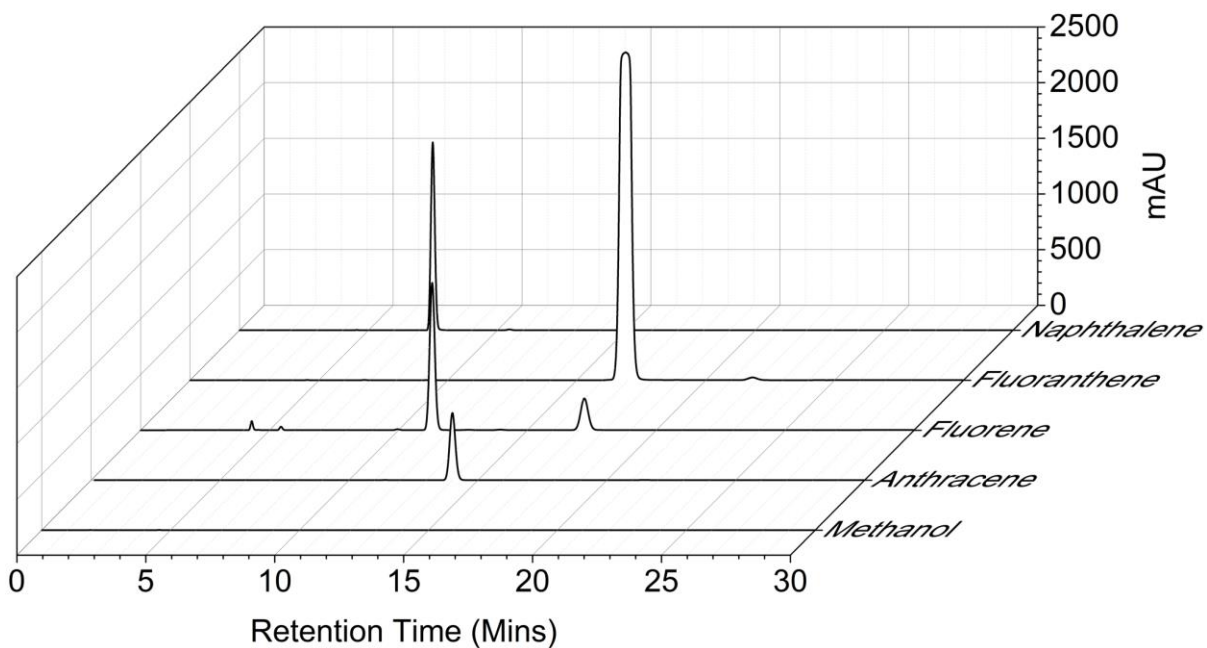


Figure 8 Standard HPLC spectrum of naphthalene, fluoranthene, fluorene, anthracene and methanol.

Table 8 Retention time of PAH standard peaks.

Name	Retention time of standard(s) (Mins)	Notes
Methanol	4.559	Major peak
Naphthalene	7.499	Major peak
Fluoranthene	16.9	Major peak
Fluorene	11.311	Major peak
	17.215	Impurities or derivatives
Anthracene	13.895	Major peak

From this series of experiments, naphthalene, fluorene and fluoranthene all have unique HPLC retention times. Therefore, these PAH compounds can be identified in other more complicated samples by examining the peaks at a certain retention time. Moreover, the concentration process performed as expected, although it might also magnify selected minor peaks caused by impurities. During the concentration process, the rotary evaporator must be cleaned before each use to avoid introduction of other substances.

4.3 Preliminary Studies with *Sphingomonas*

4.3.1 Growth Curve

The *Sphingomonas* growth curve was studied to determine whether SWCNTs adversely affect their growth. The curve also demonstrated the time required for a newly inoculated culture to achieve the stationary phase. According to the SWCNT concentrations used in membrane modification, a range from 0.01 g/L to 1 g/L is reasonable. However, due to the hydrophobic nature of SWCNTs, they were poorly dispersed even after 30 minutes of sonication. The clustered

SWCNTs caused a rapid change in the OD value in high-concentration samples (1 g/L) because the plate was shaken during cultivation, and the SWCNT chunks probably moved and blocked the OD light path at certain time points. Therefore, a relatively low concentration of SWCNT (0.1 g/L) was selected to determine the growth curve. In the desalination scenario, the amount of SWCNT exposed to bacteria is related to the concentration of unit area of the membrane, on which lower SWCNT dosage is expected. 0.1 g/L SWCNT also commonly used in this study, but higher and lower concentrations were also tested depend on the experiment design.

Figure 9 shows the growth curve of planktonic bacteria with and without the presence of 0.1 g/L SWCNTs. The similar results for the two samples indicate that SWCNTs minimally affect the growth of *Sphingomonas* at the concentration measured. This result is also verified in biofilm because the biofilm's morphology did not change even after a few days from applying SWCNT to the samples. This result should be further verified with other concentrations to confirm SWCNTs at those concentrations are not toxic to the biological component used.

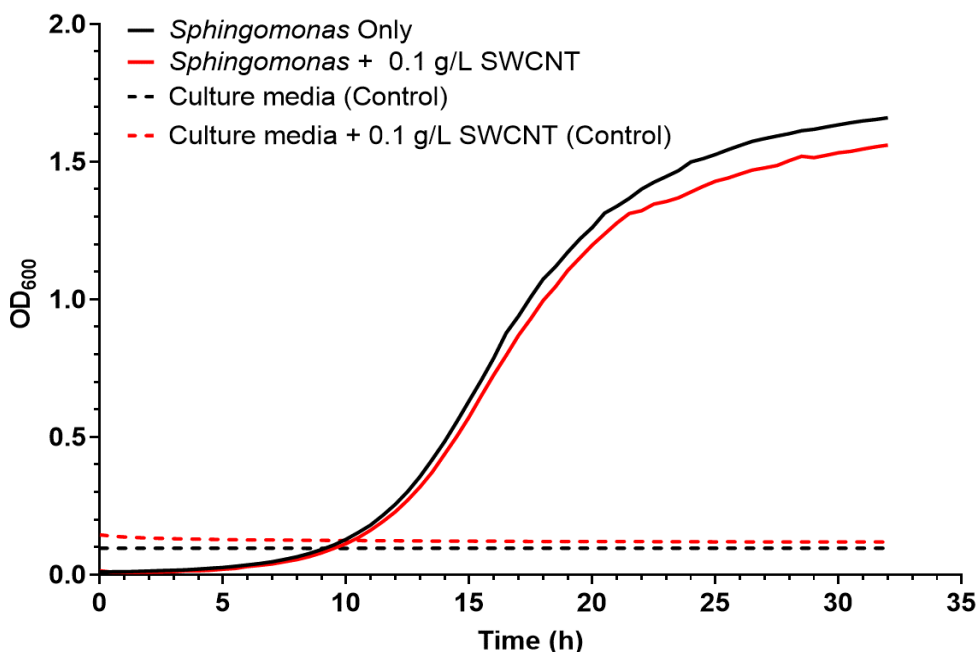


Figure 9 The 32-hour growth curve of *Sphingomonas* in TS broth at 30°C. OD₆₀₀ was measured every 30 minutes. The red solid line represents the sample with 0.1 g/L SWCNTs, and the black solid line represents the sample without SWCNTs. The control is subtracted from the curve: for bacteria only samples, the absorbance of TS broth (black dotted line) is subtracted; for bacteria + SWCNT samples, the absorbance of TS broth with SWCNT (red dotted line) is subtracted.

4.4 Studies with nitrogen sources for *N. aromaticivorans* growth

Before identifying the possible CNM DPs in the simulated SWCNT-biofilm system, it is important to gather selected basic information on biofilm metabolism and ensure that the culture condition is not adversely affect the results of degradation (all culture conditions should be verified that adequate for bacteria growth). Preliminary experiments with nitrogen sources and carbon sources were conducted and used to determine the culture media, as shown in Table 9.

Table 9 Nitrogen sources and carbon sources required for *Sphingomonas* growth. Beside the N and C sources, each sample plates contain NaCl and K₂HPO₄ to adjust the osmotic pressure. Buffer plates and agar only plates were used as control. No bacteria growth was observed on those plates (controls).

Nitrogen Source	Carbon Source	Colonies formed: Y/N
<i>Organic</i>		
Soytone	N/A	Y
Tryptone	N/A	Y
<i>Inorganic</i>		
NaNO ₃	Dextrose	N
NaNO ₂	Dextrose	N
NH ₄ Cl	Dextrose	N

The results presented in Table 9 indicate that organic nitrogen sources are not suitable for limiting the carbon source to a sole ingredient because colonies were formed even though no carbon source was added. The organic nitrogen sources, such as soytone and tryptone, consist with amino acids, which can also serve as carbon sources. Although the amount of carbon in organic nitrogen sources is significantly smaller than those in dextrose provided in this experiment, *Sphingomonas* is able to use the carbon for reproduction because the nature of it is to survive under oligotrophic conditions.

The results of inorganic nitrogen sources demonstrate that no culture was formed in the plates of nitrate, nitrite, and ammonium with dextrose and salt buffer (NaCl and K₂HPO₄). These results suggest that other nutrients, such as trace mineral elements, or the combination of inorganic nitrogen sources are essential for *Sphingomonas* growth.

4.5 Studies with *N. aromaticivorans* metabolism for aromatic compound degradation

Because the previous result indicates that *N. aromaticivorans* cannot grow in simplified media with only N, C, and P sources, more comprehensive media, namely, MSM, was used in bacteria enrichment. A crucial point in this experiment was to test whether *N. aromaticivorans* can degrade SWCNTs when they serve as the sole carbon source. Another point was to verify the capacity of the microorganisms to degrade PAHs. For all samples, the carbon source was limited to the material that was tested. The carbon source for each sample and its results are shown in Table 10.

Table 10 Result of PAH and SWCNT degradation experiment. Sample A serves as control of carbon sources; sample H serves as control of the bacteria. And sample G is Blank without any carbon source.

Sample No.	Medium	Carbon Source	Results
A (Control)	MSM	Dextrose	Colonies presence in day 2
B	MSM	SWCNT	No bacteria growth observed
C	MSM	Naphthalene	Colonies presence in day 4
D	MSM	Fluorene	Colonies presence in day 6
E	MSM	Fluorenathene	Colonies presence in day 6
F	MSM	Pyrene	Colonies presence in day 6
G (Blank)	MSM	N/A	No bacteria growth observed
H (Control)	TS Broth	N/A	Colonies presence in day 2

Colonies appeared on samples H and A after one day of cultivation. Similarly, colonies were found on sample C after 4 days. On the 6th day, colonies were confirmed on samples D, E and F. No colonies were found on samples B and G after 8 days of cultivation. The results in Table 10 indicate that the bacterium has the ability to degrade a wide range of PAHs, even those PAHs that are used as the sole carbon source. The results also indicate that *Sphingomonas* requires more time to grow on aromatic compounds than on dextrose, which suggests the most possible reason is the relatively low bioavailability of PAHs (only a small proportion of PAHs enter the bacteria's metabolism system, or the uptake is relatively slow than dextrose). Interestingly, more rapid growth was observed on the samples with fluorene compared with another PAH-containing samples. A possible explanation for this observation is attributed to the five-carbon-ring structure of fluorene, which has been reported as one type of “defect site”, and hence is easier for bacteria to attack. This result matches with findings in many other studies. In a study on the biodegradability of SWCNTs, the author notes that surface carboxylation might promote degradation⁸³. Moreover, Allen et al. also mention that carboxylated sites facilitate the degradation of SWCNTs by adsorbing enzymes such as horseradish peroxidase³⁴. However, no bacteria growth was observed on the SWCNT sample, which questions whether SWCNT is too difficult to be digested to support bacteria growth or if a cometabolism is required for SWCNT degradation.

Therefore, based on the above findings, SWCNTs with “defects” or those that have been functionalized should be further examined for their degradation capacity.

4.6 Studies with SWCNT Degradation

Following the aromatic compound degradation experiment, 0.1 g/L SWCNT and TS broth were used to determine whether SWCNT can be degraded through co-metabolism. The culture was sampled at days 0, 5, 6, and 7 and extended to days 10, 15, and 23. Figure 10 compares the HPLC spectra obtained by the extraction and concentration method. The X-axis shows the retention time, the Y-axis denotes the peak intensity, and the Z-axis indicates the days for degradation. The results from the sample with 0.1 g/L SWCNT are marked in red, and the results of the control (culture media and bacteria only) are shown in blue. Peaks with relatively small retention time were caused by the TS broth, as proved in pre-experiments.

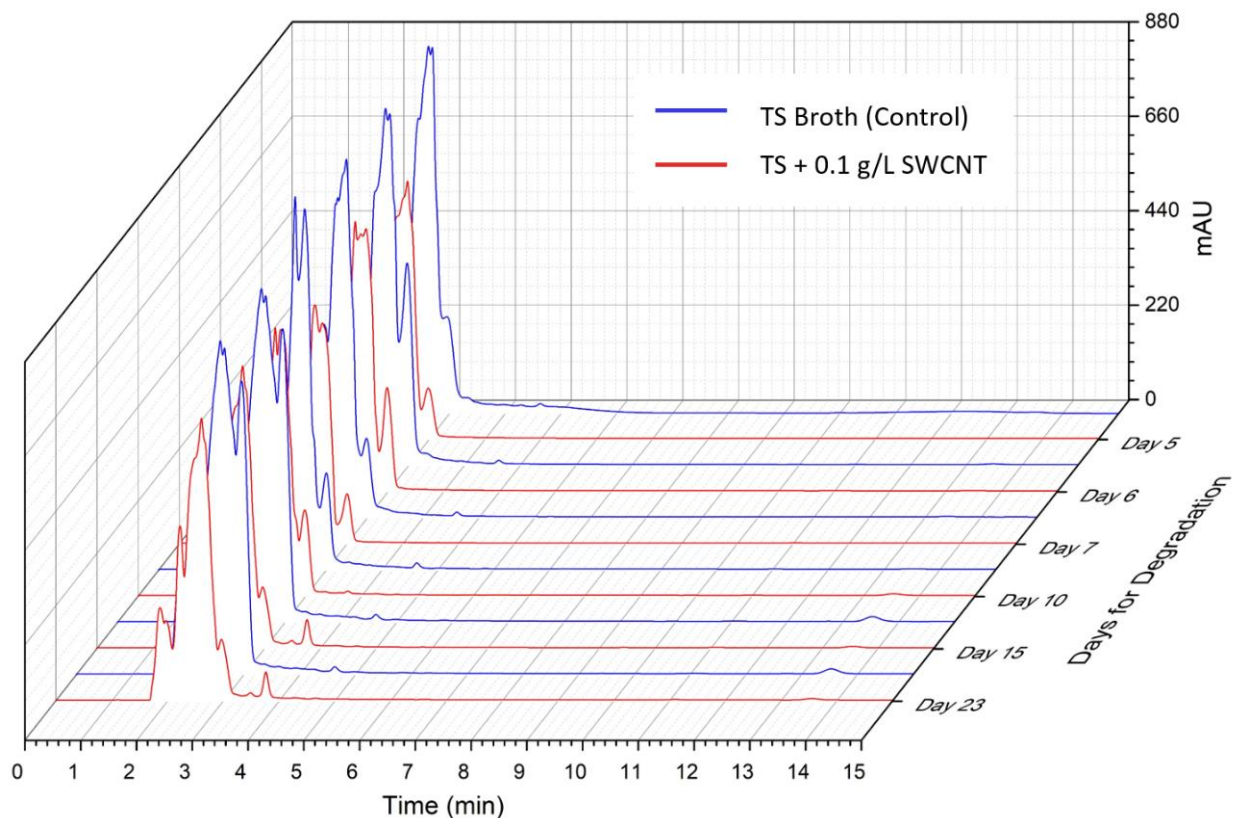


Figure 10 HPLC spectra from SWCNT degradation experiment. The X-axis indicates retention time, Y-axis denotes peak intensity, and Z-axis shows the days for degradation. Bacteria with culture media but without SWCNT server as control. The newly emerged peaks after day 10 are considered as DP but further conformation should be conducted.

The results shown in Figure 10 indicate that a new peak with a retention time of 3.76 minutes appeared after 15 days of degradation. A tiny peak is shown on the spectrum of the 10th day with the exact same retention time. Since those systematically emerged peaks are only obtained from samples with the presence of SWCNT and appeared after several days of degradation, it is highly likely that they indicate a type of SWCNT DP. Interestingly, the retention time of detected DP matches with the degradation result of PAHs (more details are demonstrated

in section 4.8.1), which also suggest that this peak is unlikely caused by experimental errors. It is possible that bacterial metabolic products and impurities, which probably caused the small peaks in control at 13.5 minutes, can affect the HPLC result, but their peak intensity cannot be as significant as the DPs due to their low concentration. Although these DPs cannot yet be identified due to the lack of mass spectrometry, the preliminary results indicate that SWCNT degradation requires co-metabolism. In a review of microbial degradation of hydrocarbon, co-metabolism is suggested to accelerate the biodegradation of heterogenous substances such as PAH because the additional carbon source supplies energy and maintains the microorganisms in an active status⁴⁴. Thus, we can speculate that the hydrophobicity of SWCNT and PAHs may need extra time to be functionalized, resulting in a slow degradation rate. In addition, co-metabolism between species might more effective for aromatic compound degradation than pure culture due to the broader enzymatic ability⁷⁸. Further assessments of the DP and co-metabolism are necessary in future study to eliminate other possibilities.

However, the results also suggest that the degradation took a long time or that at least more than 10 days are required to generate detectable DPs. The degradation efficiency might depend on many factors, but depletion of the carbon source should be the main reason for the long degradation time in this experiment. In desalination conditions, the organic matters provided by the influent could serve as the extra carbon sources. The CNM DPs are not considered as the major carbon source because the low CNM concentration in the membrane.

Based on the results, co-metabolism should be considered for future CNM degradation studies. For better degradation efficiency, external carbon sources are necessary and need to be added regularly.

4.7 Degradation Studies with SWCNTs Pretreated by Membrane Cleaning Agents

When membrane cleaning reagents are combined with established biofilm, they can serve as a complex cocktail that potentially accelerates CNM degradation by supplying electron acceptors or donors and causing defect sites on the surface of the materials. To determine their effects on SWCNT biodegradation, cleaning reagents designed for biofouling were tested. Moreover, MSM agar and TS broth were used simultaneously to further confirm the metabolism pattern for “defective” SWCNTs.

For agar plates, no visible colonies were found after 7 days of cultivation, except for the control sample (bacteria with TS broth). This result suggests that treatment by EDTA, SDS, and NaOH cannot sufficiently alter the material to support bacteria growth. But the limitation of agar plate should be noted. Since contact is required for degradation, only SWCNTs on the surface of agar plate could interact with bacteria. In order to avoid the limitation, degradation experiments in TS broth were also conducted. In the TS broth-cultured samples, although transitions of the HPLC spectrum were observed, the changes are not sufficient to be considered as DPs. In all samples, the marked peak on HPLC spectrum was shifted left about 0.3 minutes, which indicates some degree of changes happened on the starting materials. For example, additional functional group may be added onto the starting materials during degradation process and hence changed their polarity. However, further verification is required for this assumption. The HPLC results of EDTA, NaOH, and SDS are shown in Figure 11a, c, and e, respectively, and their corresponding magnifications are shown in Figure 11b, d, and f.

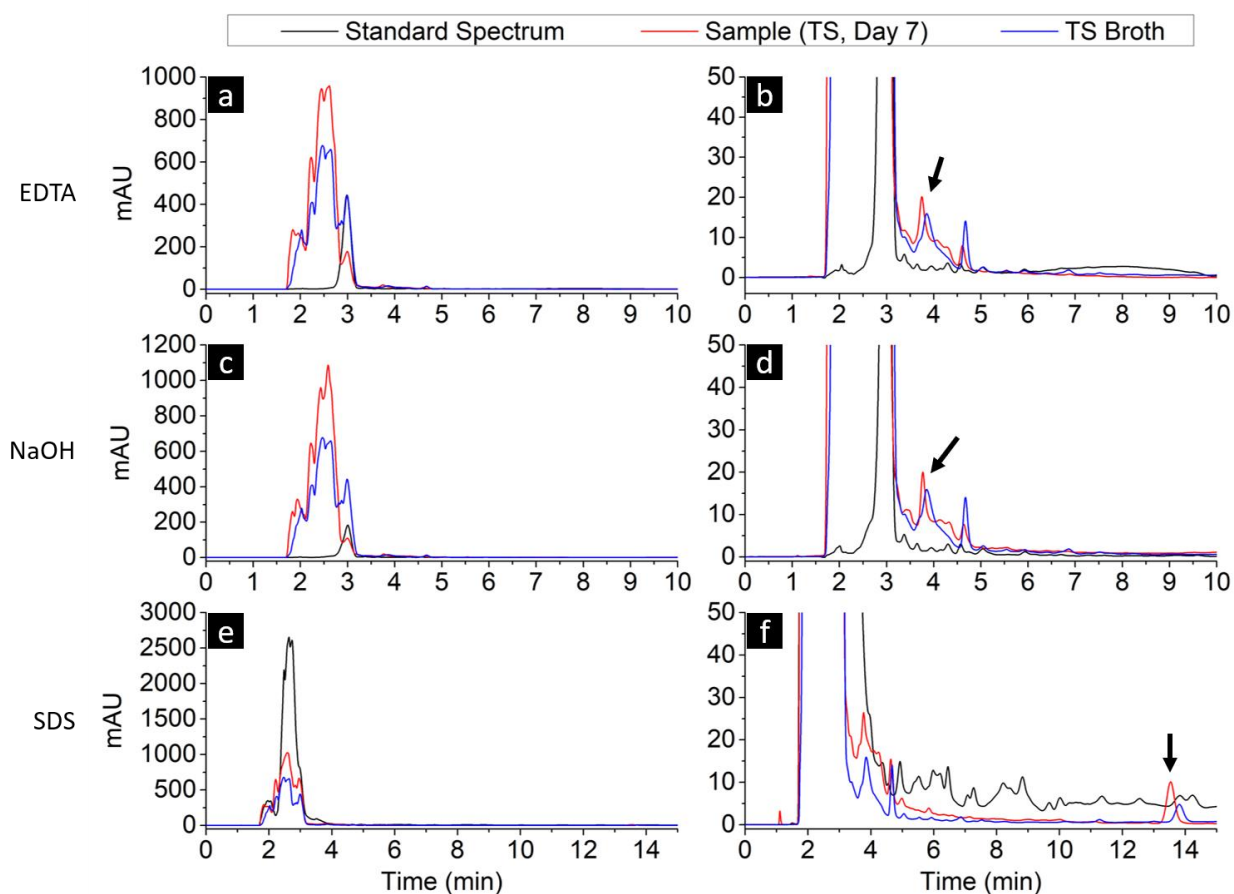


Figure 11 HPLC spectra of membrane cleaning reagent-treated SWCNT degradation experiments. Peak changes after 7-day degradation are marked with arrows. Subgraph a, c, e is the HPLC spectrum of EDTA, NaOH, and SDS, respectively. Subgraph b, d, f is the magnification of a, c, e, respectively. In the results of EDTA and NaOH, HPLC spectrum of cleaning agents treated SWCNT shifted from 3.9 to 3.7 minutes. In the result of SDS, HPLC spectrum shifted from 3.8 to 3.5 minutes.

As marked by the arrow, the newly discovered peaks are too small and are similar to the control, and thus they cannot be identified as DP. Several explanations can be considered for this result.

First, the reagents used in this experiment are not strong oxidants and might not be capable of creating sufficient defect sites on the SWCNTs. Second, microorganisms were not present during the simulated cleaning process. In this experiment, we treated SWCNTs with chemical cleaning agents first and applied the treated materials in biofilm for degradation. It is possible that cleaning agents can be used as nutrition or interact with the bacteria to promote CNM degradation. Third, the time for treatment could be too short. In this experiment, the SWCNT was treated with cleaning agents for 300 minutes based on an assumption of 10 cleaning processes of 30 minutes each. In industry, although there is no specific time period required for membrane cleaning, it may take from 4 to 8 hours. Moreover, the time intervals between cleanings could be weeks to months. Fourth, a longer degradation time may be necessary. The degradation time for this experiment was 7 days. Longer degradation time may produce more significant results. All of those considerations are suggested for future research.

4.8 Studies with DP Isolation Methods

Because PAHs and their derivatives share a polycyclic aromatic structure, they can be easily dissolved in organic solvents. Additionally, previous studies indicate that PAHs are produced through the CNM degradation process. Based on those facts, extraction is an appropriate method for separating possible CNM DPs from the environment. In fact, a similar method has been applied in various studies related to pollutant isolation. Nevertheless, limitations such as a low extraction rate for highly functionalized particles and possible loss of volatile products impede comprehensive detection of DPs. Therefore, as compensation, direct injection was developed. This section focuses on evaluating these two separation methods via PAH degradation.

4.8.1 Extraction and Concentration

Naphthalene, fluorene, and fluoranthene were examined and compared to verify the separation methods. All PAHs were applied on three-day-old biofilm and cultured for 7 more days. The PAHs and their DPs were extracted with ethyl acetate and concentrated for analysis. The results are shown in Figure 12. In addition, PAHs not involved in the degradation also underwent the isolation process to verify the validity of concentration and to determine errors that might occur.

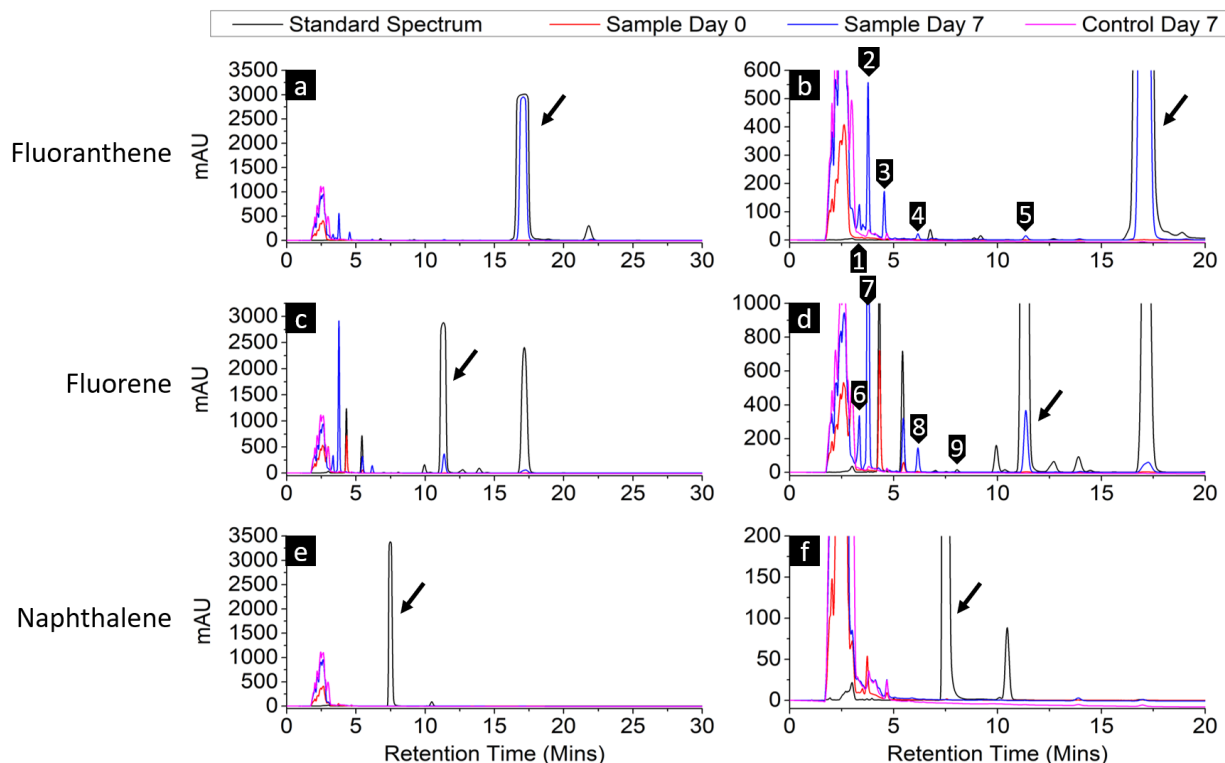


Figure 12 HPLC spectra of PAH degradation experiments. DPs are isolated by extraction and concentration method. Subgraphs b, d, and f show magnifications of a, c, and e. Parent peaks of each PAH are marked with arrows, and new peaks that appeared after 7-day degradation are labeled with a number.

Based on the spectra, significant degradation results were found in the fluoranthene and fluorene samples. The newly emerged peaks are marked with numbers. The “parent” peak of each PAH is indicated by arrows. Naphthalene is fully degraded within 7 days because no naphthalene was detected in the sample culture and no systematically emerged new peaks were found on the spectrum. 7 days should be enough to fully degrade naphthalene. In a study about naphthalene-degrading bacteria, 1 g/L naphthalene is mostly degraded after 24 hours of cultivation²⁶. The retention times of fluoranthene and fluorene DPs are summarized in Table 11 and Table 12, respectively.

Table 11 HPLC peak information for fluoranthene DPs.

Peak No.	Retention Time (min)	Comments
1	3.350	126 mAU, relatively significant peak.
2	3.774	Major Peak. 560 mAU. Probably the major/ most important degradation product
3	4.551	Relatively significant peak with peak height of 172 mAU
4	6.170	Minor peak. Around 22 mAU
5	11.361	Minor peak. Around 15 mAU

Table 12 HPLC peak information for fluorene DPs.

Peak No.	Retention Time (min)	Comments
6	3.350	336 mAU, relatively significant peak. Also found in Fluoranthene sample.
7	3.773	Most significant peak. 2916 mAU. Probably the major/most important degradation product. Also found in Fluoranthene sample.
8	6.173	Relatively significant peak with peak height of 144 mAU
9	7.012	Relatively significant peak. Around 2.5 mAU

The relatively low retention time of the newly emerged peaks suggests a strong hydrophilic property of the possible DPs, likely formed through bacterial hydrolysis, which adds hydrophilic functional groups on the material and opens aromatic rings. On the HPLC spectra of fluorene and fluoranthene, two peaks share the same retention time. This result indicates that both PAHs have the same intermediates during the degradation process and are likely to partially share a degradation pathway. In fact, one substance with a retention time of 3.773 was found in a high concentration compared with all other DPs. This compound is likely to be a highly important intermediate in the degradation process. The structural similarity between fluorene and fluoranthene might lead to its production in both samples.

Although the LC-MS system is necessary to interpret the MW and structure of the detected DPs, reviewing the literature that contains the same HPLC settings and MS information also offers helpful information on DP identification. In Feng et al.'s study on the Fenton reaction of few-layer graphene, the same HPLC parameters were used as in this study³⁵. By matching the DP spectrum with their results, three peaks were found in their results that have same retention time those as

observed in this study. The spectra and possible DPs are shown in Figure 13. Moreover, the study conducted by Allen et al. shows that DP with MW=214 (4-(2-hydroxy-1-phenylethyl) phenol) is a common compound that exists in both HRP-degraded SWCNT and FeCl₃-degraded SWCNT as well as Fenton-degraded graphene. Both researches used acetonitrile and water (with a ratio of 7:3) as solvent in HPLC. The same configuration used in HPLC and retention time between our results and literature indicate that the detected compound shares same polarity. Since it is rare that two compounds have exactly the same polarity, the DPs we detected is likely to be the same substance as the result in the literature. In addition, only qualitative result can be obtained from HPLC due to the uncertainty of DPs' structure. To further identify the DPs, mass spectrum is necessary.

Once those DPs formed, it could enter the environment (salty lake/river or ocean) with discharged brine. Along with the water cycle, they may reenter the sources of drinking water. Although the toxicity of the DPs showing in Figure 13 is not clear, it is possible for them to degrade into other particles that can adversely affect ecosystem and human health. Yet, there is no related information currently. Further research is needed to determine the relevant toxicity endpoint of the compounds and the risk posed by their degradation products.

Few layer graphene, Fenton reaction

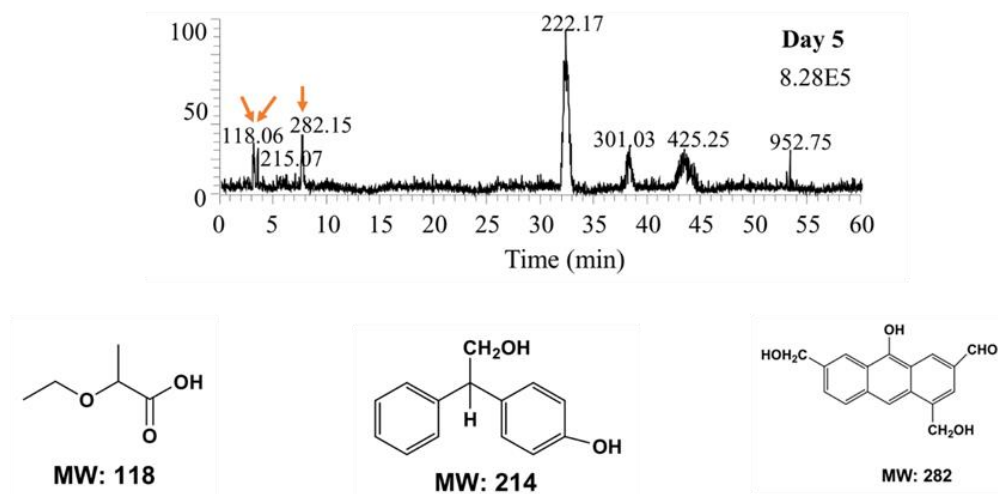


Figure 13 LC-MS results of few-layer graphene degradation, which shares the same LC specs and matches with the retention time (marked with arrow) in this study³⁵. Their retention time are 3.3, 3.7, 7.8 minutes, respectively. The DPs tend to be hydrophilic because the small retention time demonstrated.

However, the information found in other studies is limited. The literature, mostly containing MS data, does not share the exact same HPLC configuration with this study, and indeed, the previous studies do not always share the same HPLC configuration within themselves. Most differences are found in the eluent, column (type and length), flow rate, and temperature. Therefore, data in the literature cannot inform DP identification.

In addition to HPLC, studies on PAH and CNM degradation indicate that biological degradation begins with hydroxylation and results in the production of phthalic acid^{36, 37, 44, 79}. Therefore, phthalic acid could be an important intermediate within PAH degradation and is possibly produced by bacteria during this experiment. To verify whether phthalic acid was produced, its HPLC spectrum was obtained and compared with the results of the fluoranthene and

fluorene degradation experiment. The results show that phthalic acid has a similar peak shape as that of TS broth but does not match with any peaks from DP. It is possible that the degradation process is upstream of phthalic acid. Future studies on DP identification are needed to better understand the mechanisms and the degradation pathway.

4.8.2 Direct Injection

One question that emerges is whether the DPs can be lost during the extraction process, especially those that are more soluble in water. As a result of degradation, the addition of O-groups makes the DPs more hydrophilic. As a complementary method of extraction, the samples might be analyzed by direct injection into the column. The sample must be filtered to eliminate any residual biofilm or large biomolecules (e.g., filtration with a 0.2 μm filter or high-speed centrifugation).

4.8.2.1 TS Broth

Comparison of the baseline of TS broth processed by the separation methods is shown in Figure 14. Instead of a relatively flat peak, the HPLC spectrum of direct injection is sharp. The slight right-ward shift indicates that the injection volume might be slightly high because more time is needed when the ingredients exit the column. However, because it does not affect following experiments, the injection volume remained unchanged to maintain the continuity of the experiment and to make the HPLC results from different isolation methods comparable. (Injection volume of 20 μl has been used in all experiments conducted so far. Changing injection volume affects peak intensity and shapes even for same sample.) Direct injection also has simpler spectrum than the other method, in which DP can be more easily distinguished.

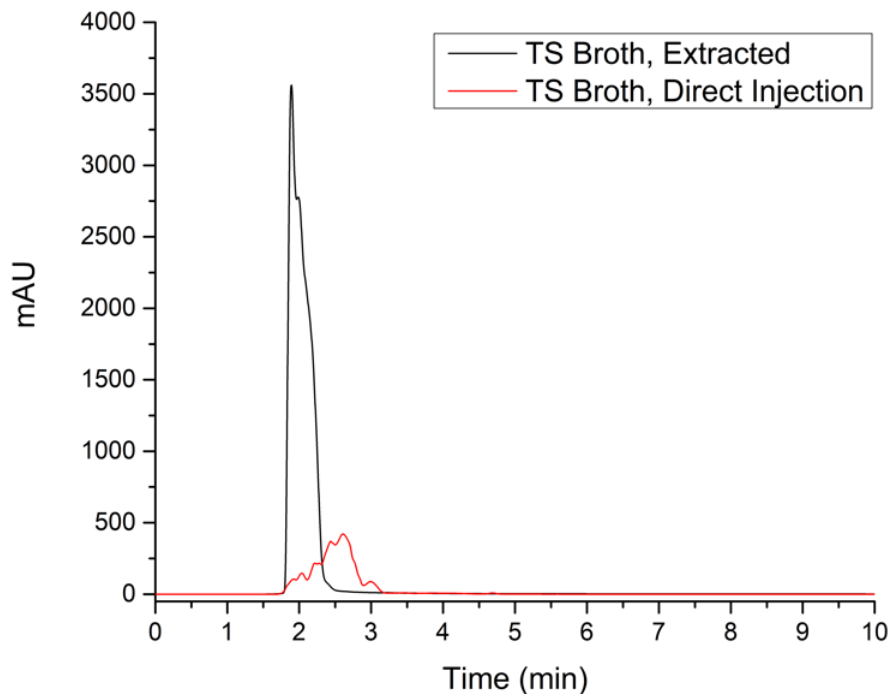


Figure 14 HPLC spectra of TS broth treated by extraction/concentration and direct injection.

PAH Samples

Naphthalene, fluorene, and fluoranthene are selected to evaluate the feasibility of direct injection. First, the “parent” peaks of each PAH were studied under direct injection conditions because any change in these peaks can be used as a reference to identify whether degradation occurred. At the same time, to confirm that PAH is not lost with this method, different agents (acetone and methanol) and filtration methods (filtration and centrifugation) were tested. The results for naphthalene, fluorene, and fluoranthene are shown in Figure 15a, b, and c, respectively

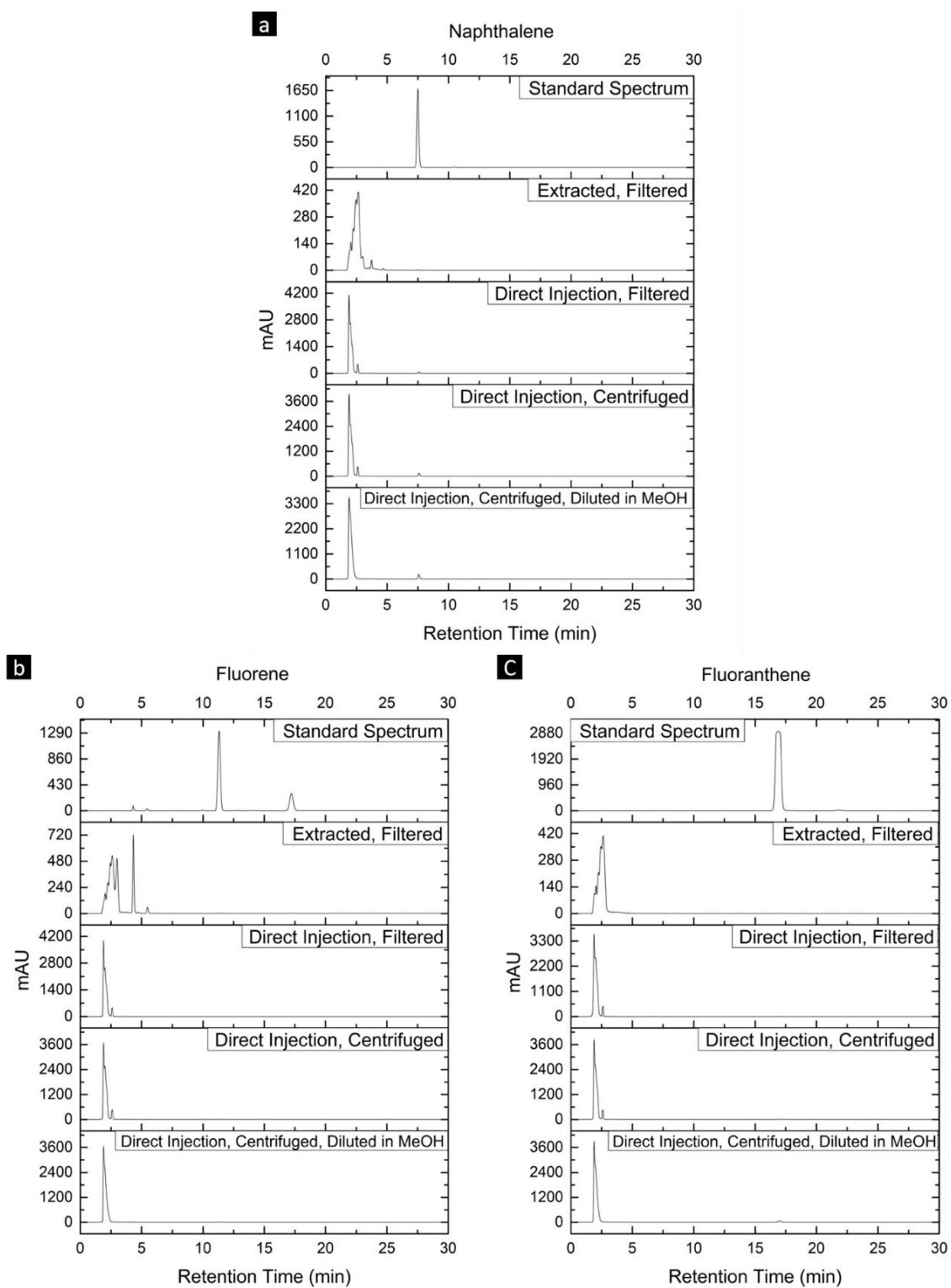


Figure 15 HPLC spectra of "direct injection"-treated PAHs. For comparison, the standard spectrum and extracted sample are included. All "direct injection"-treated PAHs were diluted in acetone unless otherwise stated. The PAHs are a) naphthalene, b) fluorene, and c) fluoranthene.

The top part of each subgraph of Figure 15 shows the standard HPLC spectra for the PAH, the filtrated sample processed with the extraction method and direct injection methods, the centrifuged sample processed with the direct injection method, and the MeOH-diluted sample processed with the direct injection method from top to bottom. Due to the notably low solubility of PAHs in the water solution, most PAH precipitate when added to the solution and are separated as insoluble particles. In specific, the solubilities of both fluorene and fluoranthene are smaller than 2 mg/L at 25°C, and the solubility of naphthalene is 31 mg/L at 25°C. Thus, their parent peaks can be barely detected. Only the naphthalene samples show a significant parent peak at a retention time of 7.59 minutes (the peak intensity is 145.7 mAU, and retention time matches with its standard peak). However, the parent peaks of fluorene and fluoranthene are notably small, with heights of 10.7 and 16.3 mAU. Therefore, direct injection might not facilitate detection of the parent peaks because PAHs have a tendency to decrease in solubility with increasing molecular weight, not to mention that CNMs are completely insoluble in water.

On the spectrum of acetone dissolved samples, a small peak can be observed at a retention time of 2.59 minutes. This peak was identified as the peak of acetone by analyzing the acetone standard spectrum and the HPLC result of the acetone-TS broth mixture. However, acetone was evaporated during the concentration process, which is why it was detected in samples processed with the extraction and concentration method.

To further evaluate this method, the reactivity of dissolved agents and TS broth with PAHs and the effect caused by filtration were also studied, and no evidence was found to indicate that any interaction occurs between them. In addition, different PAH concentrations were tested to determine the limit of detection (LOD) of the direct injection method with HPLC. Concentrations of 100 mg/L, 10 mg/L, 1 mg/L, 0.1 mg/L and 0.01 mg/L of naphthalene, fluorene, and fluoranthene

were tested. The results confirmed that concentrations below 1 mg/L are not be detectable because no PAH peaks were shown on their HPLC spectra.

4.9 Comparison of Isolation Methods

To better understand the advantages and disadvantages of these two separation methods, complete PAH degradation experiments were conducted.

4.9.1 Fluoranthene

In previous studies of fluoranthene degradation, DPs were separated from *Sphingomonas spp.* culture after 7 days by the extraction and concentration method, which suggests that fluoranthene is a reasonable choice for testing the two separation methods. However, instead of testing the possible DPs on the 7th day, they were tested every two days to further study the process of degradation. This series of data can be used to determine the time required for DPs to be generated, the process of DP transmission, and the interconnections among them.

4.9.1.1 Degradation Results

In this experiment, samples were processed with both separation methods, and the results after 7 days of degradation are shown in Figure 16.

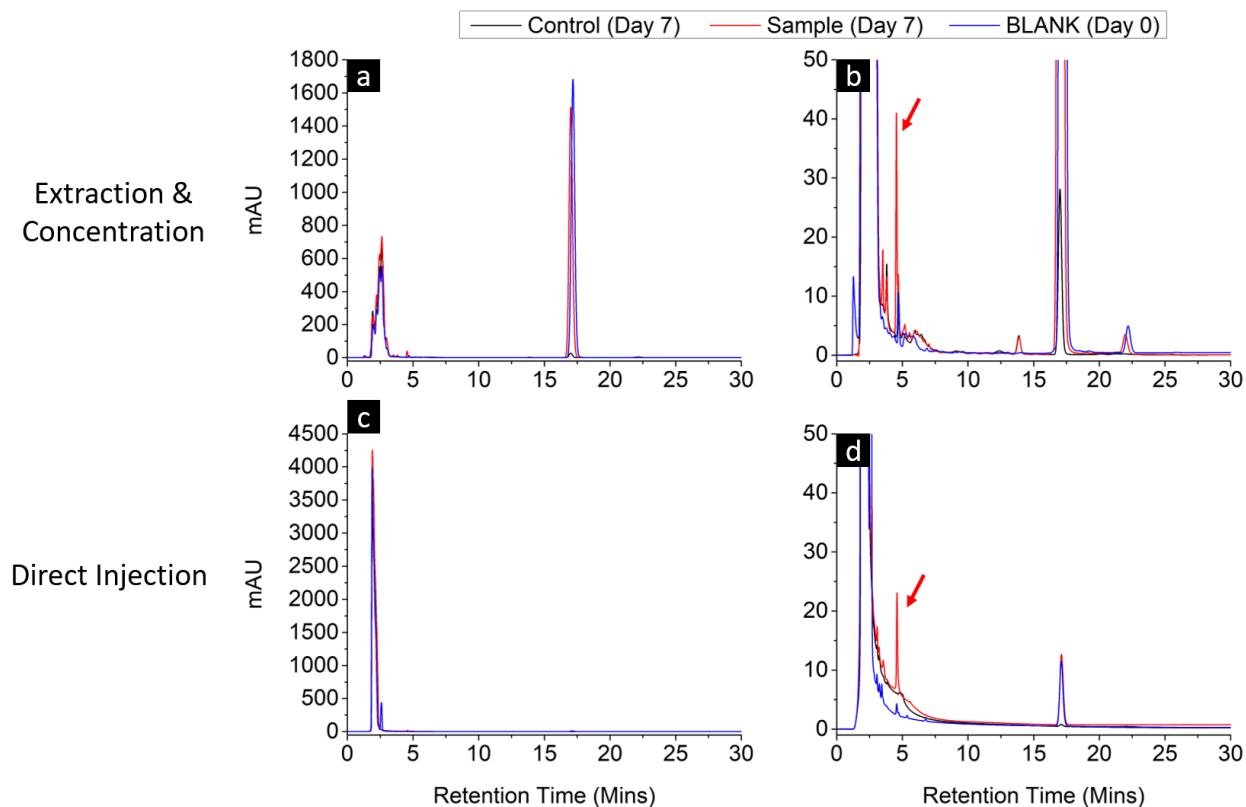


Figure 16 HPLC result of fluoranthene degradation experiments. The samples were treated by extraction/concentration (a, b) and direct injection (c, d).

According to the results obtained, fluoranthene was detected by both methods near RT = 17.0-17.1, although the peak intensity is greatly different. In the sample from the 7th day, possible degradation products are also detected by both methods (marked with red arrows). The peak with a retention time near 4.5 minutes is the most significant. The peak with such a retention time also appeared in the previous PAH degradation experiment. However, more intense peaks were observed in the previous experiment due to the higher fluoranthene concentration used. In the previous PAH degradation experiment, 1 g/l fluoranthene was applied, which is 100-times higher than the concentration used this time. In addition, the peak with a retention time of 13.9 is another

possible degradation product because it was obtained in both experiments. These results verified that fluoranthene is degradable with *Sphingomonas spp.* and that the produced DPs are consistent. However, fewer peaks were detected in the sample conducted by direct injection. The intensity of the detected peaks is prominently lower than those detected by other methods. Most detected peaks have a peak intensity within 5 mAU. However, as marked in Figure 16d, a clear peak indicates that the same DP was detected by direct injection. From this point of view, direct injection is also a viable method.

4.9.1.2 Extraction and Concentration

The DP transition in 7 days is demonstrated in Figure 17 and Figure 18. Figure 17a shows the overall HPLC spectrum of the samples treated by extraction and concentration samples through 7 days. Magnified images of the boxed regions are shown in Figure 17b, c, and d, respectively.

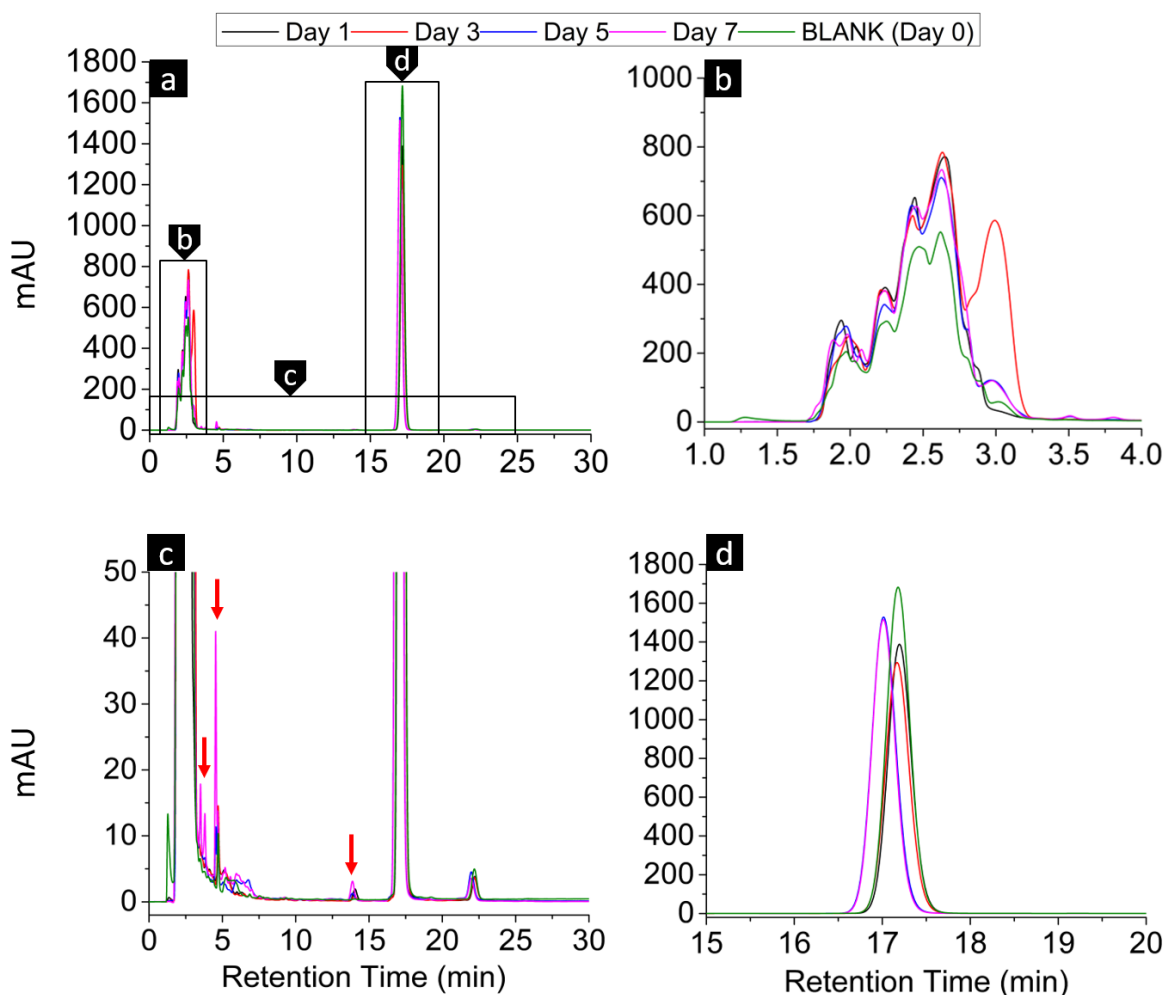


Figure 17 HPLC spectra of fluoranthene DP processed by extraction/concentration through 7 days. The magnified components in subgraph a are shown in b, c, and d. New peaks that appeared after 7 days are marked with arrows.

In Figure 17b, the HPLC spectrum of day 3 has a significant peak at an RT of 3 minutes. The peak could represent an intermediate product that was produced within the 3rd day and could be consumed thereafter. Most of the other peaks in this test are similar to those of the blank (TS broth and fluoranthene only), which indicates that no possible DP is detected in this test.

Figure 17c shows a magnification of the test c in Figure 18a, where three peaks (as marked in the graph) can be identified as possible DPs with RT values of 3.5, 4.54, and 13.87. The peaks at 3.5 and 4.54 were detected in samples from day 5 and 7, whereas the peak at 3.8 was only detected in the sample of day 7. The peaks at 13.87 were detected from all samples except the blank. Based on this result, fluoranthene could be first degraded into an intermediate with an RT of 13.87. Such an intermediate could be quickly degraded into DPs with RT values of 3.5 and 4.54. It is possible that the degradation rate of DP at 4.54 is slow, and thus it continues to accumulate over time. By the 7th day, one or both of the DPs are further degraded to the DP at 3.8.

Figure 17d gives the magnification of the fluoranthene peak. On day 5 and day 7, the peak was slightly shifted to the left, which might indicate that changes occurred in the fluoranthene. This peak could indicate that a new functional group was added onto the molecule, such as hydroxy, which therefore changed its polarity, but it could also suggest structural changes such as carbon ring opening.

Generally, extraction and concentration work well for DP separation, amplifying the concentration of DPs and demonstrating the results more clearly, but it is possible to introduce impurities and experimental errors.

4.9.1.3 Direct Injection

The overall HPLC spectra of samples processed by direct injection are shown in Figure 18a. Magnified views of the framed region are shown in Figure 18b and c.

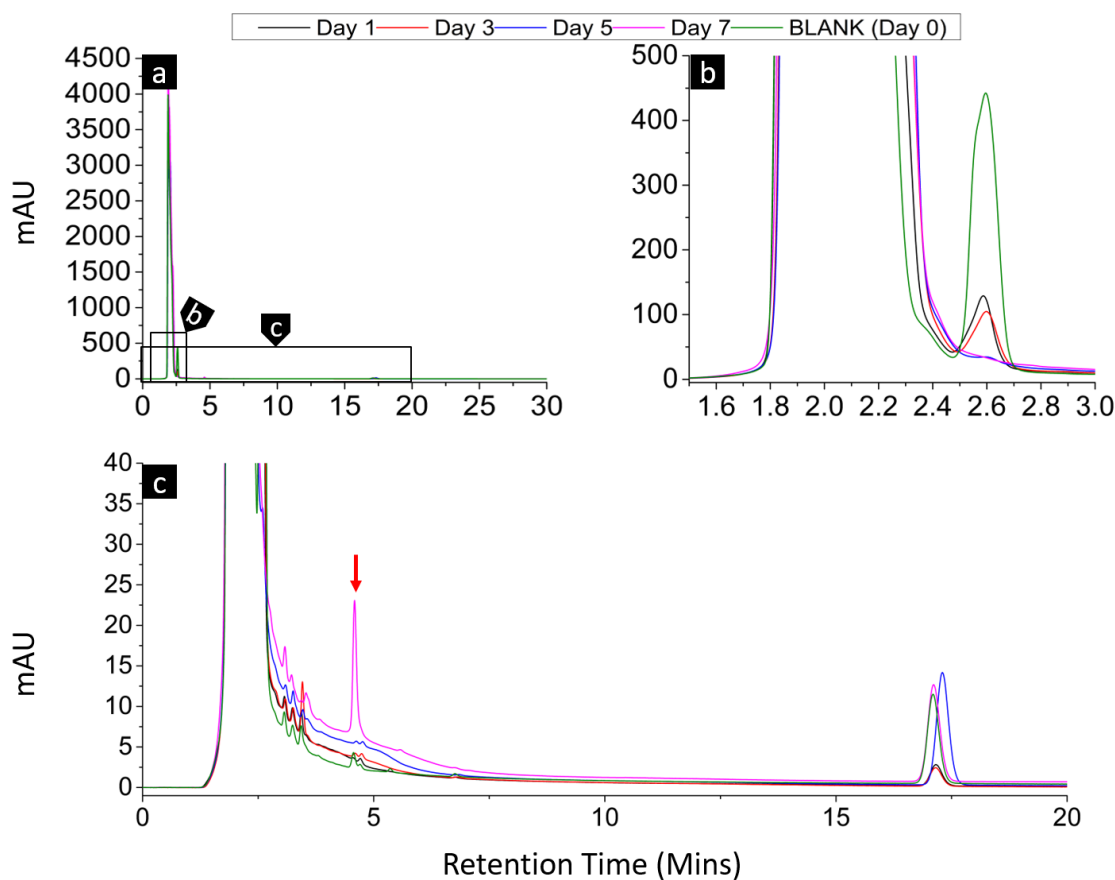


Figure 18 HPLC spectra of fluoranthene DP processed by direct injection through 7 days. The zoomed components in subgraph a) are shown in b) and c). New peaks that appeared after 7 days are marked with arrows.

A decreasing peak can be observed in Figure 18b and represents acetone based on a previous HPLC spectrum of the TS broth-only sample and TS broth-acetone sample. Acetone was used to dissolve fluoranthene and is chosen for better PAH dispersion. The decrease in acetone is caused by either evaporation or bioutilization and is undetectable after 5 days of degradation.

The peak of a possible DP was found at RT of 4.5 minutes (marked in Figure 18c). However, this peak appeared on the seventh day, indicating that no significant degradation might occur before this point. A more notable observation is the change in the fluoranthene parent peak at an RT of 17.3. Although the intensity is low on the first and third day (which is consistent with the fact that PAH is not easily soluble in water), it increases in the following days and is slightly shifted, which matches the results from the extraction and concentration method and suggests that fluoranthene might be modified during degradation.

In summary, both methods demonstrated the ability to detect the DP of fluoranthene. Direct injection offers ease of operation, but the peak strength is significantly lower than that of the other method. In this experiment, only one peak for possible DP was observed. However, the extraction and concentration method is more complicated, and it is reported that this method might lose low-molecular-weight particles during concentration and could also magnify the error caused by impurities. However, additional possible DP peaks were found in this experiment, and it also demonstrates a clear “parent peak” of fluoranthene.

4.9.2 Anthracene

To further verify the separation methods, anthracene was selected as the target PAH because it is structurally the same as CNM, and its derivatives have been reported to be present in the CNM degradation process. Based on the fluoranthene degradation result in which DPs were detected on the 7th day, a longer degradation time was applied. An amount of 10 mg/L anthracene in TS broth was also measured on day 0 of the experiment as the blank sample. This sample supplies information for comparing the differences before and after bacterial degradation. Possible

DPs were sampled and analyzed every two days. For the direct injection samples, two replicates were sampled every two days to avoid error caused by contamination.

4.9.2.1 Degradation Results

The results after 11 days of degradation are shown in Figure 19. Figure 19b and d show magnified regions of Figure 19a and c.

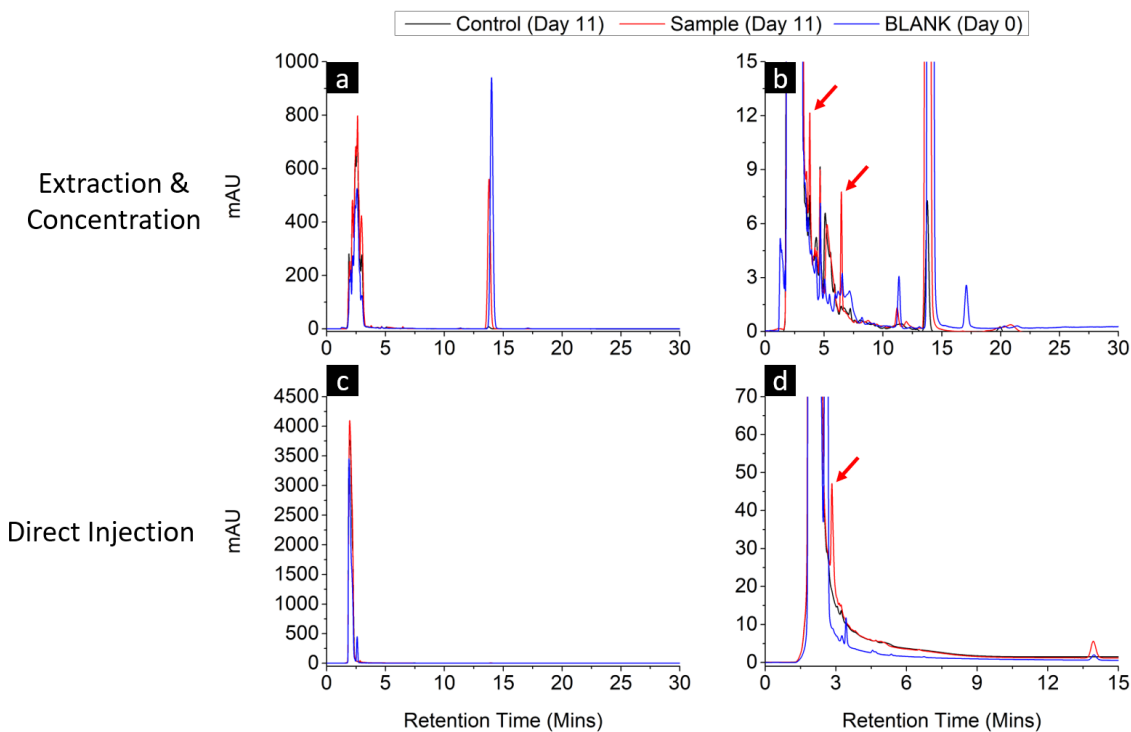


Figure 19 HPLC result of anthracene degradation experiments. The samples were treated by extraction/concentration (a, b) and direct injection (c, d). DP peaks after 11 days are marked with arrows.

According to the results, the parent peak of anthracene was detected by both methods near RT = 13.4-14, although the peak intensity is greatly different. Possible degradation products are also detected by both methods (marked with arrows). In the extraction and concentration-treated samples, the peak with a retention time of 6.5 is probably caused by degradation products. In the direct injection samples, the emerging peak at 2.9 could represent degradation products. Fewer peaks were detected in the direct injection samples. The intensity of these detected peaks is prominently lower than those detected by the other method.

4.9.2.2 Extraction and Concentration

The HPLC spectra from day 1 to day 10 are shown in Figure 20.

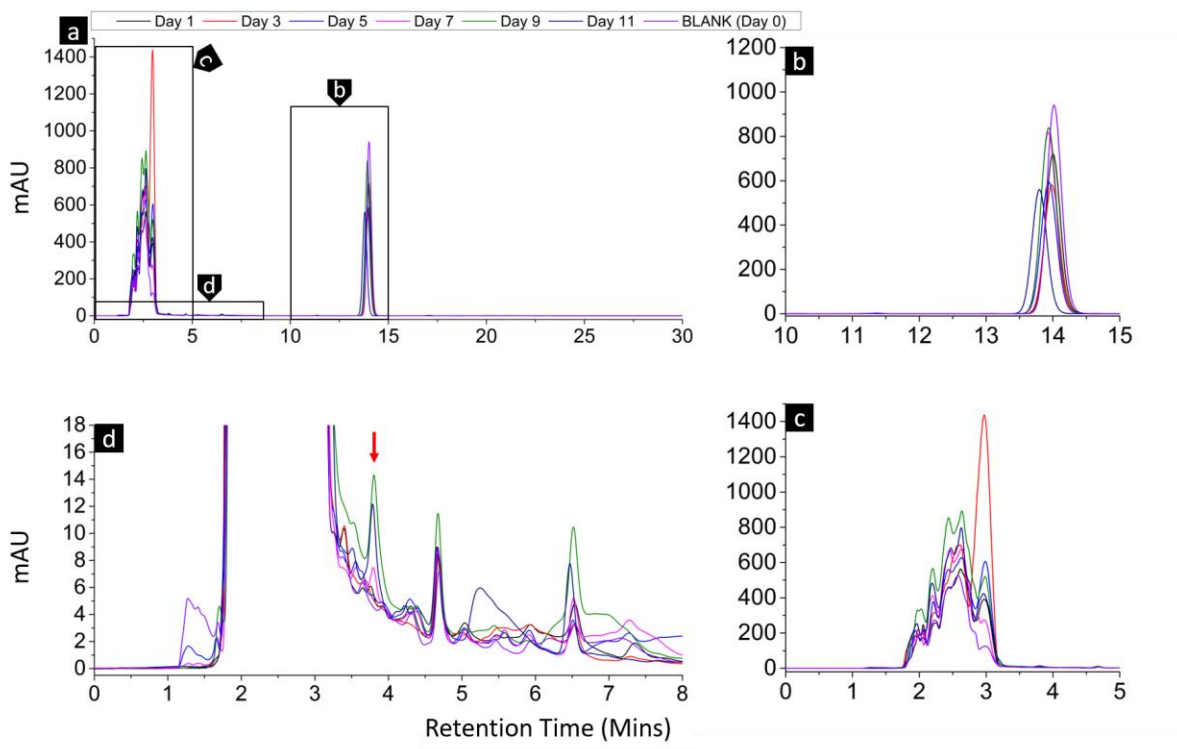


Figure 20 HPLC spectra of anthracene DP processed by extraction/concentration through 11 days.

The HPLC spectrum of day 3 has a significant peak at an RT of 3 minutes in Figure 20. As shown in Figure 20c, the peaks with a retention time near 3 minutes increase in intensity through the first 3 days and subsequently decrease in the following days. Because the same tendency was observed in the controls, these peaks are unlikely to be caused by degradation products. One possible suggestion is that such peaks with a retention time of 3 are caused by certain metabolic products from the *Sphingomonas*. If this is true, it indicates that the *Sphingomonas* grew rapidly within the first 3 days and that growth slowed after the nutrients in the broth were consumed. This explanation is possible because such a peak was not detected in the blank without bacteria. In addition, these peaks were also detected in the previous fluoranthene degradation experiment. The strength of the peak on the third day has the highest value.

In Figure 20d, the peak of anthracene was shifted to the left with time. This shift could be related to a new functional group that was added onto the anthracene, such as hydroxy, via the degradation process and therefore changed its polarity. This change could also represent structural changes such as carbon ring opening.

Figure 20d presents a magnified view from 0 to 8 minutes of the spectrum. In this view, the peak with a retention time near 3.8 could be identified as a possible DP because it increased over days and was not detected in the blank and control.

4.9.2.3 Direct Injection

The direct injection samples are shown in Figure 21.

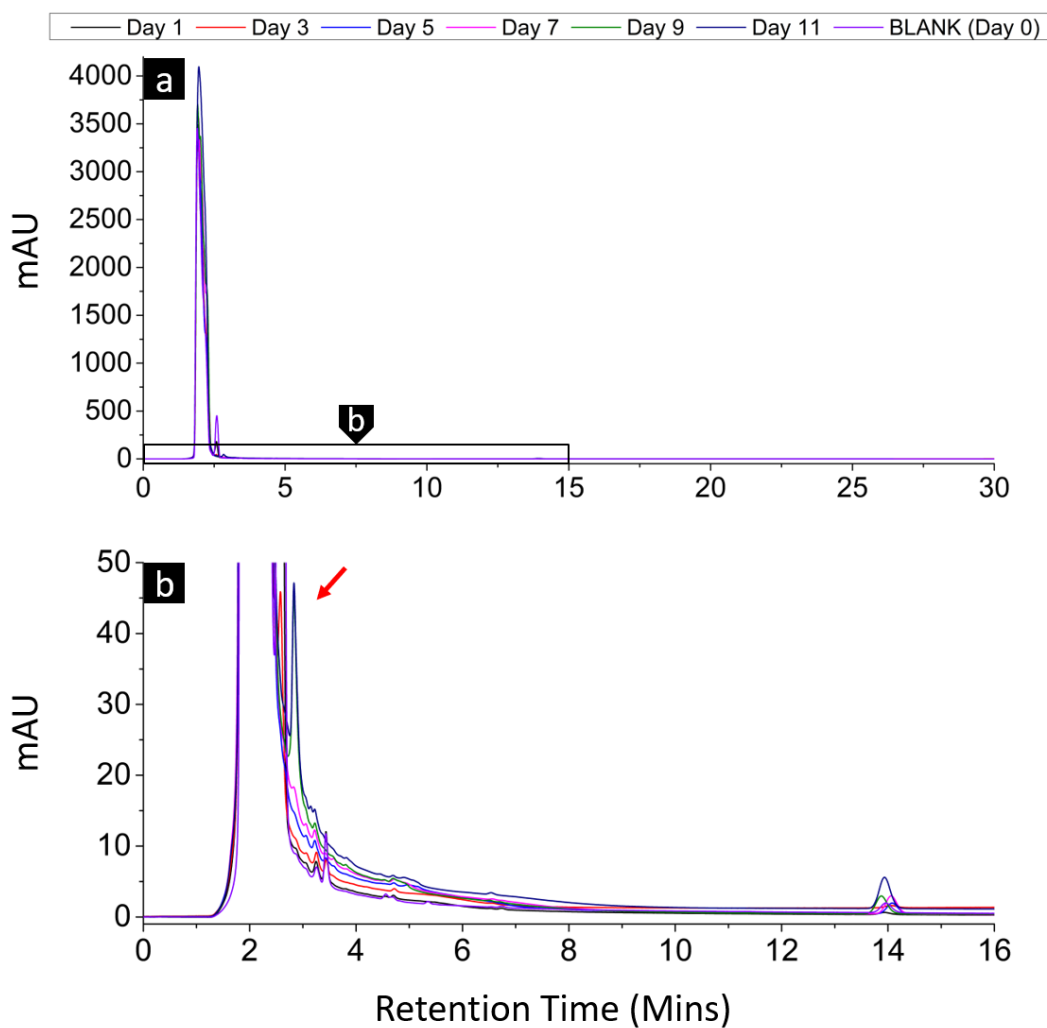


Figure 21 HPLC spectra of anthracene DP processed by direct injection through 11 days. The DP peak is marked with an arrow.

Only one DP was detected in the spectrum. In Figure 21b, a peak at an RT of 2.9 appeared after 7 days of degradation, which suggests that the degradation of anthracene might require more time than other simpler PAHs such as fluoranthene. No “parent” peak for anthracene was obtained in the first 3 days because of the notably low solubility of anthracene in water solution (0.044 mg/L at 25°C). However, the peak for anthracene was detected in the samples after 3 days of degradation, which suggests that the solubility of anthracene might be changed through the degradation process. For example, the addition of a hydrophilic functional group might result in a higher dissolution ratio. It should be noted that the parent peak of fluoranthene was detected because it has relatively high solubility (0.265 mg/L at 25°C).

The low peak strength of possible degradation products detected by both methods indicates a low degradation rate of anthracene. Because the molecular weight of anthracene is less than that of fluoranthene (178.23 to 202.26 g/mol), anthracene should be easier to degrade. One possible factor for limited degradation could be the viability of the bacteria. In this experiment, the biofilm disintegrated after 6 to 7 days of culture. Without exchanging the broth, nutrients could be easily consumed in a short time period, and thus the number of bacteria might decrease over time. Apparently, the low concentration of anthracene is insufficient for bacteria growth. Based on the results, the broth should be changed within a certain period of time to maintain the vitality of the bacteria. The growth curve of *Sphingomonas* suggests that 2 days might be a feasible time period for changing broth.

In addition, the differences among the different PAHs degraded and CNMs suggest that their degradation pathway are not completely the same, but the common results on retention time may indicate a partly shared degradation mechanism. In both metabolism and cometabolism, the target compound is required to be functionalized or break down for bacteria digestion. The random

nature of the process results in the production of DPs with different polarity, which cause different retention time in HPLC results. Also, it should be noticed that both methods cannot measure SWCNT parent peak because its insolubility in water and organic solvent. To prevent any damage caused by insoluble particles, SWCNTs must be filtered prior to the injection.

For separation methods, both methods demonstrated the ability to detect the DP of anthracene. Direct injection offers ease of operation, but the peak strength is lower than that of the other method. However, the extraction and concentration method is more complicated, and it is reported that this method might lose low-molecular-weight particles during concentration and could also magnify the error caused by impurities. However, additional possible DP peaks were found in this experiment, and it also demonstrates a clear “parent peak” of anthracene.

5.0 Summary and Conclusion

The primary objectives of this study were to develop methods that support the investigation of CNM DP formation in membrane water treatment conditions. A preliminary literature review supported the establishment of an experimental approach that included (i) SWCNTs as one of the base CNM structures, (ii) *Sphingomonas spp.* as a primary bacterium-formed biofilm on the RO membrane, and (iii) standard alkaline chemicals used in the membrane cleaning processes. The premise for the simulated environment is that over time, desalination membranes develop chemical (e.g., mineral scale) and biological (e.g., biofilm) build-up and eventually require aggressive cleaning to restore the process efficiency. The established biofilm itself or the combination of biofilm and membrane cleaning agents possibly degrades SWCNTs and PAHs. The DP detected in this study showed that the use of *Sphingomonas* (specificly *N. aromaticivorans*) for SWCNT and PAH degradation is effective in terms of their role in forming biofilm and its ability to digest aromatic compounds. The isolation methods used in this study, including extraction and direct injection, were proven to be effective for separating possible DPs from a complex mixture. The ability to separate and detect DPs from a complicated simulated desalination system is promising for future DP identification and risk evaluation studies. Overall, the results from this study show that the degradation of SWCNTs is affected by the metabolic environments that provided for the bacteria, the treatment with introduced chemicals, and the bacteria culture conditions (e.g., time and medium). However, more comprehensive assays are required to identify the possible DPs and assess their hazards in the environment and the human body.

The specific conclusions obtained in this study can be summarized as follows:

1. The degradation of SWCNTs and representative PAHs (naphthalene, fluorene, fluoranthene, anthracene, and pyrene) by *Sphingomonas* in biofilm form is demonstrated.
2. SWCNT cannot serve as the carbon source for *Sphingomonas*. An additional carbon source (e.g., dextrose, is necessary to observe degradation under the conditions studied), which suggests a co-metabolic degradation process. In real desalination conditions, hydrocarbons in the inflow might serve as the additional carbon source for CNM degradation.
3. Cleaning reagents used in this study, including sodium hydroxide, EDTA, and SDS, demonstrate limited ability to affect SWCNT degradation, which could be due to the following: (i) SWCNTs were treated with a cleaning reagent before contacting the bacteria, (ii) only alkaline agents were applied in this study, and/or (iii) the growth media were not regenerated within the timespan, which could result in limited bacterial metabolic activity at longer time points. For the first point, the chemical agents involved in the cleaning process might combine with the established biofilm, serving as a viable mixture to degrade the SWCNT. For the second point, in addition to the alkaline agents used in this study, acids (e.g., HCl and citric acid), oxidants (e.g., NaOCl and hydrogen peroxide) and other chemicals (e.g., rhamnolipid) are used in membrane cleaning. Those agents might represent more possibilities to promote the degradation of SWCNTs due to their ability to supply protons or peroxy radicals. For the third point, continuous supplement of nutrition (C, N sources and minerals) is necessary for the bacteria to maintain viability and consequently to maintain the ability to cometabolize SWCNT.

4. This study evaluated two isolation methods for DP detection: (i) extraction and concentration and (ii) direct injection. Although both methods work well for DP isolation, they have different advantages. The extraction and concentration method contains more processing steps than direct injection, which has higher resolution to characterizing potential degradation of the parent material. However, direct injection offers a more facile approach to detecting possible hydrophilic DPs because the extraction agent used in the other method might result in loss of those compounds due to their low solubility in an organic solvent. In general, the two methods can complement each other for more comprehensive detection results.
5. The common retention times of HPLC peaks among the three PAHs studied herein suggest that they might share DPs and thus partial degradation pathways. DPs represented by the common HPLC peaks suggest that they probably are important intermediates that serve as “principal” compounds within the degradation process.

6.0 Suggestion for Future Research

Several areas that would be worthwhile to pursue regarding the identification of possible DPs and analysis their potential hazard on environment and human health.

1. Further DP identification should be conducted using additional analytical procedures, such as mass spectrometry (e.g., LC-MS). The current study was limited to HPLC methods to determine the conditions under which degradation occurred and was unable to obtain the specific chemical structure information of the formed DPs. Material characterization techniques, such as XPS, TEM, and Raman spectroscopy, can complement the mass spectrometry data and confirm changes in the CNM structure and surface chemistry before and after degradation.
2. Comprehensive viability assays should be applied for biofilms to assess the effect of bacterial growth on the degradation process. The viability assay can provide quantitative analysis on bacteria number, which can be used for evaluating the health of biofilms and determine the dynamic change of bacteria during degradation process. It should be noted that the MTT assay is incompatible if SWCNTs are present in the sample because of their ability to interact with the major constituent of tetrazolium salts⁸⁵.
3. Since CNMs are often functionalized to enhance binding to the membrane^{58, 63}, the effect of functional groups on CNM degradation should also be investigated. Moreover, carboxyl- and amine-functionalized CNMs were reported to be more susceptible to biological degradation. Hydrophilic sites introduced by carbonylation can interact with the active site of enzyme (e.g., the heme active site of horseradish peroxidase). The

oxygen-containing and nitrogen-functionalized defective sites demonstrate a shorter time for enzyme degradation^{42, 81, 83}.

4. Further exploration of the degradation characteristics of different types of CNM structures (MWCNT and graphene) should be conducted to evaluate the contribution of morphology to the production of DPs. Comparison of the DPs across CNMs might inform studies of the degradation pathway.
5. Additional membrane cleaning reagents should be tested in future studies. Oxidants such as sodium hypochlorite and hydrogen peroxide should particularly be applied due to their ability to supply protons or peroxy radicals. Such peroxy radicals can bind with the active site of enzymes (e.g., hydrogen peroxide), serving as a crucial constituent for the enzyme in reaction with SWCNTs³⁴.
6. The test system should be scaled-up to approach more realistic conditions (e.g., by isolating *Sphingomonas* or other relevant biofilm-forming bacteria from environmental samples).

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